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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The objective is to design, build and study vectors which would be able to break tolerance to breast cancer associated TAA and be used to suppress the recurrence of metastatic breast cancer following surgical resection. The hypothesis is that by fusing the CD40 ligand stripped of its transmembrane domain and intracytoplasmic domains, to a breast cancer TAA such as the extracellular domain of the her-2-neu receptor, or the extracellular tandem repeat peptides of breast cancer associated surface glycoprotein, MUC-1 (both of which have been shown to be capable when loaded on APCs of conferring resistance to engraftment by cancer cells bearing these TAA), one can break tolerance to breast cancer. The subcutaneous injection of this vector creates infected cells as factories to secrete the CD40LTAA into the systemic circulation as well as locally for the activation and antigen loading of APCs, so that they would move to the lymph nodes all over the body to generate a CD8 dependent response against metastatic breast cancer. We also explored boosting of the vector vaccine by TAA/CD40L protein injections. This report summarizes the successful assembly and study of these vectors. These injections break tolerance to tumor associated antigens in mouse models.

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adenoviral vector, gene therapy, metastatic breast cancer, Her-2, Neu, MUC-1

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A. Introduction.

This is a report of the results of the research for the period July 15, 2003-July 14, 2004. The goal of the studies was to test the effect of subcutaneous injections of TAA/CD40L vectors and proteins in mice which were anergic to the TAA. The immune response ignores breast cancer cells because 1) they are covered by self antigens which are present from birth and therefore are indistinguishable from normal tissue and 2) the surveillance cells of the immune response (dendritic cells (DCs) do not have access to tumor cells. We have designed a method (see Figure 1A) for in vivo activation and tumor antigen loading of dendritic cells (DCs) so that they can induce a T cell immune response against tumor associated antigens (TAA) in cancer (1-2). The ultimate goal is to develop this vaccine to reduce the recurrence rate following definitive local therapy of breast cancer patients who are at high risk of recurrence.

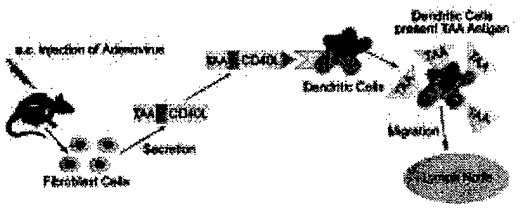
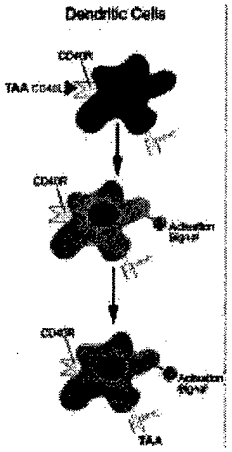


Figure 1A

B. Body.

B.1. Approach for In Vivo Activation and Tumor Associated Antigen (TAA) Loading of DCs. An adenoviral vector which carries a fusion gene composed of the TAA fused to the extracellular domain (ecd) of the CD40 ligand (CD40L), is injected subcutaneously (sc). The cells infected by the vector are programmed to secrete the TAA/ecdCD40L protein for 10 days. When the CD40L end of the protein binds to the CD40 receptor on the DCs, the DCs are activated to take up the TAA (see Figure 1B below) and migrate to the regional lymph nodes. The DCs, which the carry pieces of the TAA on the MHC molecules on their surface, induce an expansion of the T cells which are capable of recognizing and killing TAA positive cancer cells, and making antibodies to the TAA. We found that this vector vaccination can induce immune suppression of the growth of TAA cancer cells for up to one year even in mice which are anergic to the TAA (1-2).We have studied 3 different TAA in mouse models: the HPV E7 antigen, the rat Her-2-Neu receptor, and the hMUC-1 antigen. We have chosen on to use the hMUC-1 antigen to develop this program.

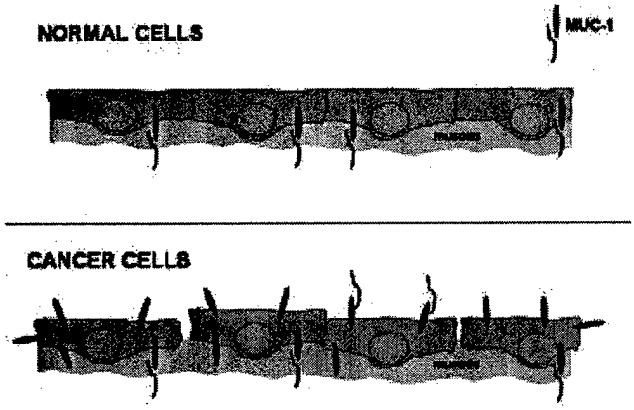
Figure 1B



B.2. MUC-1 Antigen. The MUC-1 antigen (3-6) is a structural protein which is expressed at very low levels on the apical surface of epithelial cells thereby protecting the mucosal surfaces by stabilizing the mucous covering on epithelial surfaces in secretory ducts (breast, prostate) and on other mucosal surfaces (gastrointestinal, respiratory, cervical, and endometrial). As shown below in Figure 1C, although the protein is translated as a single protein, it is cleaved into two subunits: a transmembrane protein with a 65 amino acid (AA) intracytoplasmic domain and a 69 AA extracellular domain, and a second subunit which consists of a large (up to 1800 AA) extracellular protein. This latter protein carries a large but variable number of highly glycosylated repeat domains of 20 AA each associated with the ecd of the transmembrane protein through non covalent interactions (5-6).

Although the basic function of MUC-1 in epithelial neoplasms remains that of protection of the cells of the epithelial surface, many differences are acquired that distinguish MUC-1 in cancerous epithelia surfaces from epithelial surfaces in normal tissues (6-7). As shown in Figure 1C, there is an increase in the density of the transmembrane subunit on all surfaces (not just the apical surface) of the epithelial cell. Most but not all of the transmembrane proteins are unassociated with the larger extracellular subunit. Both the large and smaller transmembrane subunits are hypoglycosylated in the neoplastic epithelial cells thereby exposing tumor specific epitopes in the cancer cell (4-7).

Figure 1C



The over expression of the MUC-1 protein results in translocation of this protein into the outer membrane of the mitochondrion and changes in the WNT pathway, which results in increases in the resistance of the cancer cell to DNA damage induced cell death by radiation and chemotherapy (6). Finally, the overexpression of MUC-1 in cancer cells disrupts the E cadherin pathway, resulting in loss of intercellular bridges, increased motility of the cells and increased tendency to metastasize (7-8). MUC-1 is found to be overexpressed in above 70% of all epithelial neoplastic cells, including carcinomas of the breast, lung, prostate, ovary, cervix, endometrium, esophagous, stomach and colon (3-4).

Based on this data, we created a chimeric cDNA composed of two tandem repeats from the 1800 AA extracellular domain subunit which was linked to the aminoterminal end of the extracellular domain of the CD40L, from which the transmembrane domain had been removed. This cDNA was inserted with a signal sequence down stream of the CMV promoter in the E1A region of an adenoviral vector (1-2) from which the E3 gene had been removed (Ad-sig-ecdhMUC-1/ecdCD40L). We also created similar vector and protein vaccines for using the Her-2-Neu receptor (Ad-sig-rH2N/ecdCD40L).

B.3. Overview Summary of Results.

We accomplished the following:

1. Completion of the assembly of the Ad-sig-rH2N/ecdCD40L vector and study of the effect of subcutaneous injections of the Ad-sig-rH2N/ecdCD40L vector into rH2N.Tg mice.

2. Study of the effect of different schedules of Ad-sig-hMUC-1/ecdCD40L vector and hMUC-1/ecdCD40L protein into the hMUC-1.Tg mice.

The conclusion was that a single Ad-sig-TAA/CD40L vector followed by two TAA/CD40L protein subcutaneous injections break tolerance to TAA, induces increases in the cellular and humoral immune response, and induces an immune response in the transgenic mice that recognize TAA on human tumor tissue. These results suggest that this vaccine may be of value in reducing recurrence rates of breast cancer following initial definitive regional therapy.

Detailed Summary of Results. (The Specific Aims refer to those cited in the Statement of Work in the Original Application).

B.4. Specific Aim #1: Study of Various Ad-TAA/CD40L Vectors to Test the Effect of Engineering a Secretable or Non-Secretable TAA/CD40L Transcription Unit. This work has been completed and is summarized in the publications provided in the Appendix (Zhang et al, and Tang et al). The result is that the TAA/CD40L transcription unit must be secretable. Therefore, all subsequent studies were carried out with secretable TAA/CD40L transcription units.

B.5. Specific Aims #2-3: Construction and Detailed Study of Vector Vaccine for Human MUC-1 Tumor Breast Cancer Associated Antigen. One of the TAA chosen for this work was the human MUC-1 (hMUC-1) antigen in hMUC-1.Tg mice, which are transgenic for the hMUC-1 and have been reported to be anergic and unresponsive to stimulation with hMUC-1 antigen (9). The MUC-1 is a "self antigen" that has been shown to be diffusely over-expressed as a neo-antigen (due to an unique pattern of glycosylation) in 80% carcinomas of the

breast, colon, endometrial, ovary, pancreas and prostate (11). The overexpression of the MUC-1 antigen is thought to disrupt the E-Cadherin system and thereby lead to metastases (6-8). In contrast to the cancerous epithelial surfaces in which the MUC-1 protein is diffusely overexpressed, the MUC-1 antigen is barely detectable only on the apical borders of normal secretory epithelia cells.

Results. When Dr. Y. Tang of the Deisseroth lab injected the Ad-sig-hMUC-1/ecdmCD40L vector sc into the hMUC-1.Tg transgenic mice, which are immunologically unresponsive (9) to human MUC-1 positive mouse syngeneic cancer cells (LL2/LL1hMUC-1), the hMUC-1.Tg mice became resistant to the growth of the hMUC-1 positive LL2/LL1hMUC-1 tumor cells (see Figure 2). This resistance was specific for the hMUC-1 antigen (see Figure 3), and the survival of these mice increased dramatically (bold line in Figure 4 below) compared to unvaccinated mice (broken thin line in Figure 4.

Figure 2

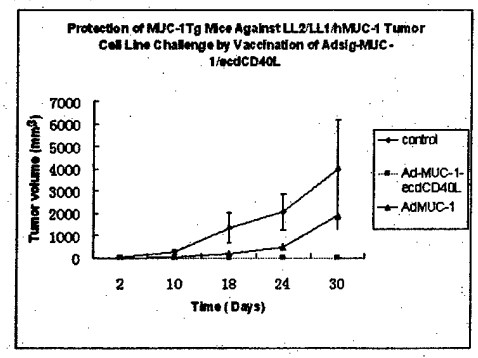


Figure 3

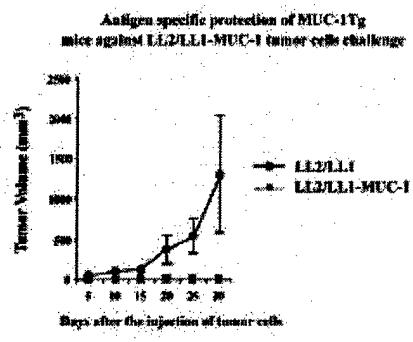
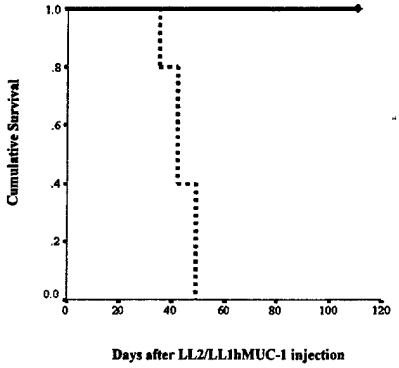


Figure 4



B.6. Specific Aims#2-3: Vector Vaccine for Her-2-Neu Positive Breast Cancer Cells. The overexpression of the Her-2-Neu (H2N) growth factor receptor in 30% of breast cancers is associated with increased frequency of recurrence after surgery, and shortened survival. In order to test if the Ad-sig-TAA/ecdCD40L vaccination strategy could break tolerance for this self antigen, we vaccinated mice which were transgenic for the rat H2N (rH2N) gene and therefore were tolerant of this gene with the Ad-sig-rH2N/ecdCD40L vector (19). As shown below in Figures 5-7, sc injection of the Ad-sig-rH2N/ecdCD40L vector: 1) increased the level of rH2N specific interferon gamma positive splenic T cells (see Figure 5); 2) increased the level of antibodies against rH2N (see Figure 6); and 3) induced resistance to the growth of a syngeneic cell line which was rH2N positive (see Figure 7).

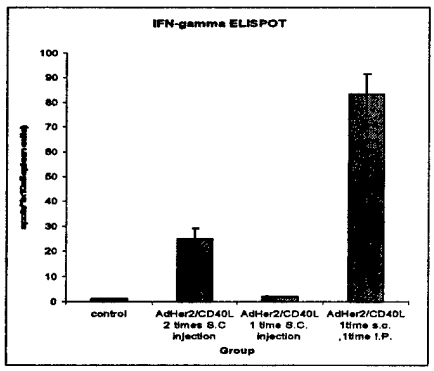


Figure 5

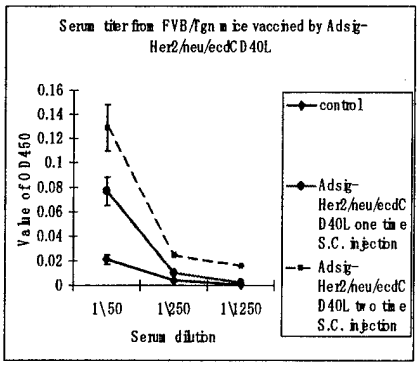


Figure 6

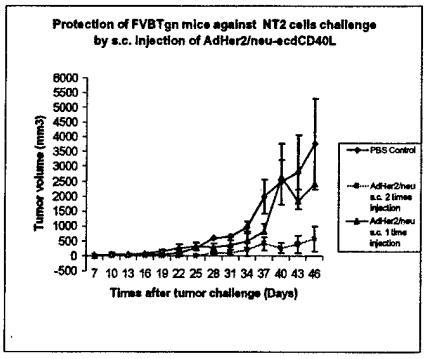


Figure 7

B.7. Specific Aims #3-4: Protein Boosting for Vector Vaccine. We next tested if the sc injection of TAA/CD40L protein could induce an immune response in TAA anergic animals, without first being vaccinated with the Ad-sig-TAA/ecdCD40L vector. As shown below in Figure 8, the sc injection of the TAA/ecdCD40L protein itself without previous Ad-sig-ecdhMUC-1/ecdmCD40L vector injection, could not totally suppress the growth of the hMUC-1 tumor cells in the hMUC-1.Tg mice. We then tested various combinations and schedules (Table I) of the Ad-sig-ecdhMUC-1/ecdmCD40L vector and ecdhMUC-1/ecdmCD40L protein sc injections. As shown in Figures 9-10, vaccination with the Ad-sig-ecdhMUC-1/ecdmCD40L vector followed at 7 and 21 days with sc injection of the ecdhMUC-1/ecdmCD40L protein (T5 in Table I and Figs. 9-10), induced in the vaccinated hMUC-1.Tg mice the biggest increase (of any of the schedules in Table I) in the level of hMUC-1 specific splenic T cells (Figure 9), and 2) increases in the level of hMUC-1 specific antibodies (Figure 10).

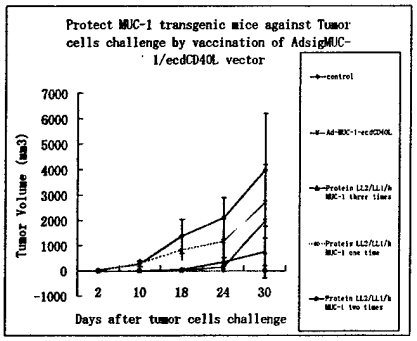


Figure 8

Vector then Protein Boost

Table I

Testing Group	Week 1	Week 2	Week 3	Week 4
Control	**Vector**	**Vector**	Nothing	Nothing
Treatment 1 (T1)	**Vector**	**Vector**	**Protein**	Nothing
Treatment 2 (T2)	**Vector**	**Vector**	Nothing	**Protein**
Treatment 3 (T3)	**Vector**	**Protein**	Nothing	Nothing
Treatment 4 (T4)	**Vector**	Nothing	**Protein**	Nothing
Treatment 5 (T5)	**Vector**	**Protein**	Nothing	**Protein**
Negative Control	Nothing	Nothing	Nothing	Nothing

Table I

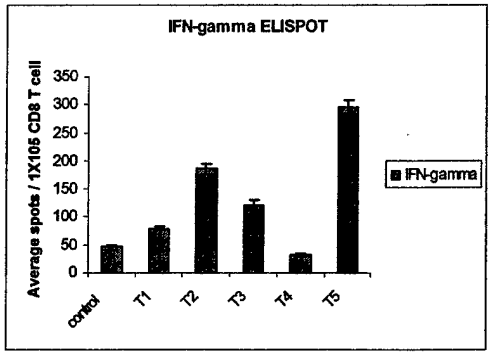


Figure 9

This schedule of one Ad-sig-ecdhMUC-1/ecdmCD40L vector injection followed in 7 and 21 days by subcutaneous injections of the ecdhMUC-1/ecdmCD40L protein led to complete suppression of the growth of the hMUC-1 positive cancer cells (see VPP in Figure 11). This has led to our proposing the clinical trial shown above in Figure 1D. This has been reviewed by the NIH RAC, the local IBC and approved by the local IRB.

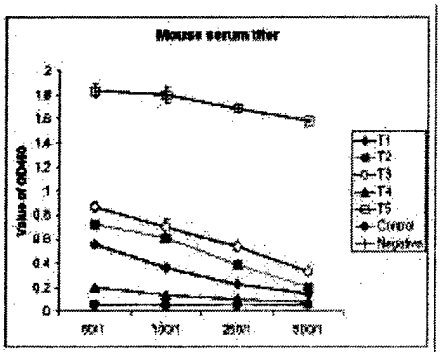


Figure 10

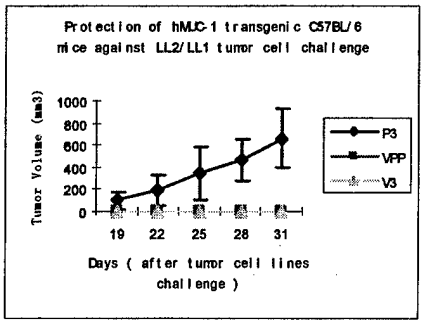
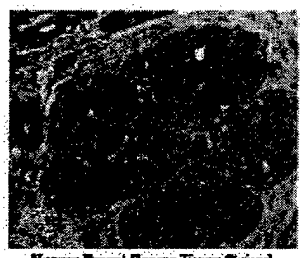


Figure 11

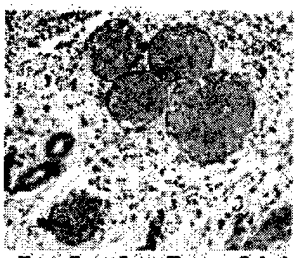
B.8. Staining of Tissue Microarrays of Normal Human Breast Epithelial Cells and Human Breast Cancer Cells in Tissue Microarrays Using Serum from hMUC-1.Tg Mice Before and After Vaccination with the Ad-sig-ecdhMUC-1/ecdmCD40L Vector.

Hypothesis and Goal. A hypothesis that could be tested is that the antibodies which are generated using the VPP vaccination program shown above in Figures 10-11 (one Ad-sig-ecdhMUC-1/ecdmCD40L vector and two ecdhMUC-1/ecdmCD40L protein subcutaneous injections) is that the hMUC-1 specific antibodies which are generated by this vaccination program would react with biopsies of normal breast epithelial tissue and breast cancer epithelial tissue. We have therefore tested if these antibodies bind to tissue microarrays which contain biopsies from normal and neoplastic epithelial cells.

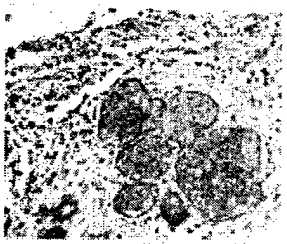
Method. Dr. Tang of our laboratory purchased tissue microarrays which contained biopsy specimens from normal and neoplastic breast epithelial cells were exposed to the serum from the Ad-sig-ecdhMUC-1/ecdmCD40L vector and ecdhMUC-1/ecdmCD40L protein vaccinated mice (VPP). After washing, the slides were then exposed to a horse radish peroxidase (HRP) secondary antibody which recognizes mouse IgG antibody. As control, these antibodies were exposed first to a hMUC-1 peptide from the antigenic repeat of the hMUC-1 domain which is used for the vaccination.



Human Breast Cancer Tissue Stained with Serum from Vaccinated Mice



Human Breast Cancer Tissue was Stained with Serum from Unvaccinated Mice



MUC-1 Peptide Inhibits Staining of Human Breast Cancer Tissue by Serum from Vaccinated Mice

Figure 12

Results. As shown below in the left hand panel of Figure 12, the mouse IgG from the vaccinated mice bound to the breast epithelial cells in the biopsy specimens of cancerous epithelial cells. There was no binding to the intervening fibroblast or stromal cells in the biopsy specimens indicating that the antibodies specifically recognized breast epithelial cells (see left upper panel of Figure 12). The fact that the serum from the unvaccinated mice did not react with the breast epithelial cells suggests that the antibodies are induced in the mouse serum by the hMUC-1/ecdCD40L vaccination.

In order to test if the antibodies were directed to the hMUC-1 repeat, a peptide containing the aminoacid sequence from the hMUC-1 repeat was added in stoichiometric excess to the antibody before the antibody was layered on the tissue microarray. As a control, a scrambled peptide was added to the antibody. As shown in the left lower panel of Figure 12, the hMUC-1 peptide blocked binding of the mouse IgG from the vaccinated mice to the breast cancer epithelial cells.

Analysis and Conclusions. The Ad-sig-ecdhMUC-1/ecdmCD40L vector and ecdhMUC-1/ecdmCD40L protein induce an antigen specific response which is specific for the repeat sequence of the extracellular domain of the breast epithelial cells and this antibody cross reacts with human breast cancer cells as predicted by Figure 1B. This suggests that the vector prime/protein boost vaccination will induce a hMUC-1 specific humoral and cellular response in human patients against the breast cancer epithelial cells.

B.9. Specific Aim #3: Optimal Regimen for Prevention-Vaccination Before Subcutaneous Injection and Tail Vein Injection of hMUC-1 Positive Cells in Syngeneic Immunocompetent Mice.

Goal. hMUC-1.Tg mice were vaccinated with various combinations of the Ad-sig-ecdhMUC-1/ecdmCD40L vector and ecdhMUC-1/ecdmCD40L protein. The Ad-sig-ecdhMCU-1/ecdmCD40L vector was injected and followed at 7 and 21 days by subcutaneous injection of the ecdhMUC-1/ecdmCD40L protein (VPP) or this schedule using the Ad-sig-ecdhMUC-1/ecdmCD40L vector for all three injections (VVV), or using three injections of the ecdhMUC-1/ecdmCD40L protein for all three injections (PPP) in order to test which of these vaccinations could best protect these mice which were anergic to the hMUC-1 antigen from the growth of the LL2/LL1hMUC-1 cell line.

Method. hMUC-1.Tg transgenic mice were injected subcutaneously with the Ad-sig-ecdhMUC-1/ecdmCD40L vector on day 0 and then the ecdhMUC-1/ecdmCD40L protein at 7 and 21 days following the vaccination with vector (VPP) or VVV or PPP. Animals were injected subcutaneously with 1.5×10^5 LL2/LL1hMUC-1 cells. At the same time, the mice were injected through the tail vein with 1.5×10^5 LL2/LL1hMUC-1 cells. The serum was collected from the animals at 14 days after the vector vaccination for study of the levels of hMUC-1 specific antibodies and. Animals were also followed for the size of the subcutaneous tumors. Finally at 28 days after the injection of the LL2/LL1hMUC-1 cell line, the animals were sacrificed and then the following studies were carried out: 1) levels of hMUC-1 antibodies in the serum, and 2) the weight of the lungs (as a measure of the growth of metastatic lung cancer) from the LL2/LL1hMUC-1 cells injected into the tail vein.

Results. Tumor Growth Curve. As shown in Figure13A, there is little or no suppression of tumor growth from the PPP schedule, whereas both the VVV and the VPP schedules protect the hMUC-1 mice from the growth of the LL2/LL1hMUC-1 cell line.

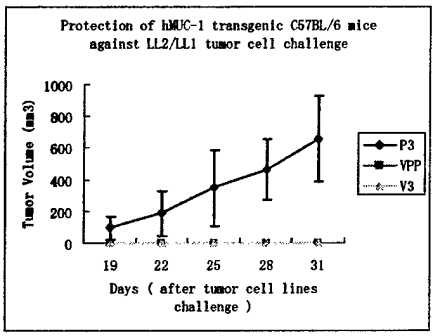
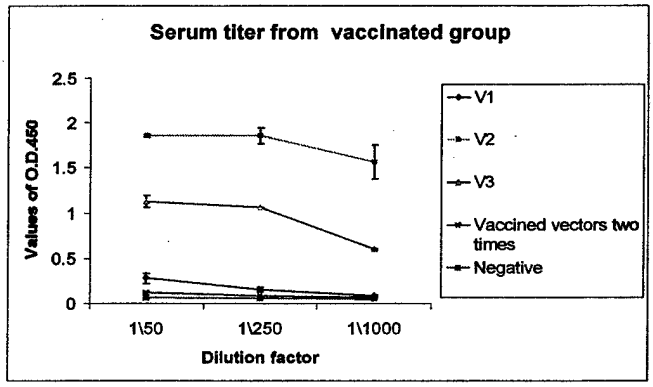


Figure 13A. Growth of Subcutaneous Nodules of the LL2/LL1hMUC-1 Cell Line.

Results. hMUC-1 Specific Antibody Levels. As shown in Figure 13B, the hMUC-1 antibody levels were determined on serum which was collected from the mice 14 days after the vaccination was completed. The hMUC-11 specific antibody levels are highest in the hMUC-1.Tg mice vaccinated with the VPP schedule (V2 or solid squares in Figure 13B).

Figure 13B



Results. Lung Weights. In order to test how effective the three regimens of vaccination were (VVV, VPP, and PPP), the hMUC-1.Tg mice were sacrificed at 42 days after completion of the vaccination (which is 28 days after the tail vein injection of the LL2/LL1hMUC-1 cell line). As shown below in Figure 13C, the mice injected with the VVV and VPP were completely protected from the growth of the tail vein injected LL2/LL1hMUC-1 cells in the lung, whereas the PPP vaccinated mice received no protection at all.

B.9. Summary and Conclusions of the Experiments for Vaccination of hMUC-1.Tg Mice. The vaccination with the VPP schedule is superior to the VVV or the PPP as measured by the levels of hMUC-1 specific antibodies which are generated at 14 days after the completion of the vaccination. The PPP schedule of vaccination does not protect the hMUC-1.Tg mice at all from the growth of the LL2/LL1hMUC-1 cell line either injected subcutaneously or intravenously. Thus, in the protection experiment, the VPP appears to be the preferable protocol.

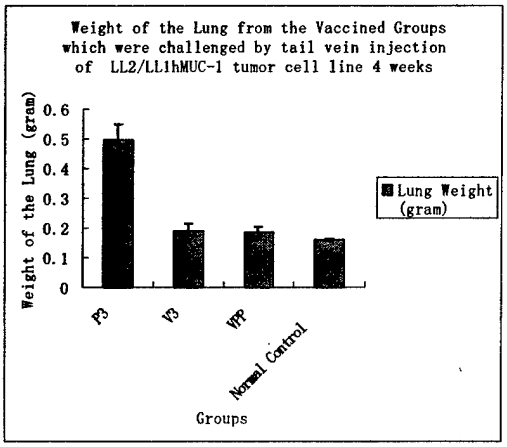


Figure 13C

B.10. Specific Aim #4: Optimal Regimen for Therapy. Vaccination Following Subcutaneous and Tail Vein Injections of the hMUC-1 Positive Tumor Cells into Syngeneic Immunocompetent hMUC-1.Tg Mice.

Goal. These experiments were designed to test whether the VVV, VPP or PPP vaccination schedule, administered following the injection of the LL2/LL1hMUC-1 cell line subcutaneously and 30 days after intravenous injection of the LL2/LL1hMUC-1 cell line could induce regressions or control the growth in the hMUC-1.Tg mice of the syngeneic LL2/LL1hMUC-1 cell line.

Methods. hMUC-1.Tg mice were injected subcutaneously and intravenously with the LL2/LL1hMUC-1 cell line at day 0 and day 30 days respectively. Then these mice were vaccinated with various combinations of the Ad-sig-ecdhMUC-1/ecdmCD40L vector and ecdhMUC-1/ecdmCD40L protein. The Ad-sig-ecdhMCU-1/ecdmCD40L vector was given 7 days after the initial subcutaneous injection of the LL2/LL1hMUC-1 cell line followed at 7 and 21 days later by subcutaneous injection of the ecdhMUC-1/ecdmCD40L protein (VPP) or this

schedule using the Ad-sig-ecdhMUC-1/ecdmCD40L vector for all three injections (VVV), or using ecdhMUC-1/ecdmCD40L protein for all three injections (PPP) in order to test which of these vaccinations could best induce regressions of the LL2/LL1hMUC-1 cell line in these mice which were anergic to the hMUC-1 antigen.

Results. Antibody Levels. As shown in Figure 14A below, the levels of the hMUC-1 specific antibodies were highest in the hMUC-1.Tg mice which were vaccinated with the VPP schedule (T2 or solid squares). The other two regimens (VVV or PPP) were only slightly higher than the unvaccinated animals.

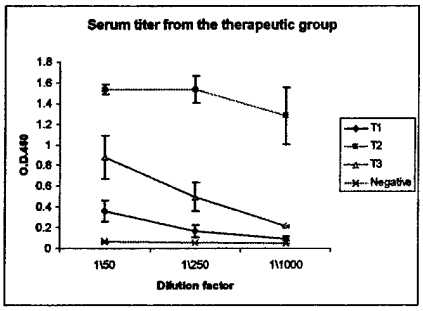
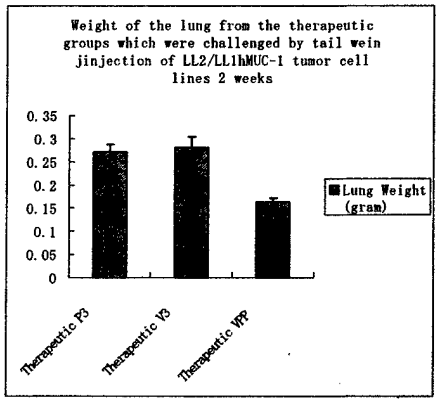


Figure 14A. T1(VVV group) T2 (VPP group) T3 (PPP group)

Results. Tumor Growth Curve: The growth of the subcutaneous tumor cell line LL2/LL1hMUC-1 after the scinjection of the three vaccine programs: VVV, VPP, and PPP, is least with the VPP vaccination program (Data not shown). The growth is most rapid after the PPP vaccination program.

Figure 14B



Results Lung Weights. As shown above in Figure 14B, the growth of the LL2/LL1hMUC-1 cell line when injected intravenously, as measured by tumor that is growing in the lung, is least with the VPP vaccination schedule.

Conclusions. The VPP vaccination program is the best as measured by: hMUC-1 antibody levels, suppression of subcutaneous nodules of LL2/LL1hMUC-1 cell line and the suppression of the intravenously injected LL2/LL1hMUC-1 cell line as measured by lung weights.

B.11. Summary Conclusions. The Ad-sig-TAA/ecdCD40L vector vaccine has been shown to break tolerance to TAA in two transgenic mouse models in which the mice are immunologically unresponsive to the TAA. This work is summarized in papers from the Deisseroth laboratory (1-2). Since the Ad-sig-ecdhMUC-1/ecdmCD40L vector and ecdhMUC-1/ecdmCD40L protein vaccine strategy has been approved by the local IRB, and reviewed by the local IBC and the NIH RAC, it is logical to develop a phase I clinical trial for toxicity evaluation. Ultimately, our goal is to add this clinical vaccine to standard adjuvant therapy in the post surgical adjuvant setting in patients who are thought to be at high risk of recurrence despite conventional adjuvant therapy.

C. Key Research Accomplishments.

1. The Ad-sig-ecdhMUC-1/ecdCD40L vector subcutaneous injections have been shown to break tolerance in hMUC-1.Tg mice which are previously anergic to hMUC-1.
2. The Ad-sig-rat-Her-2-Neu/ecdCD40L vector subcutaneous injections have been shown to break tolerance in rat Her-2-Neu transgenic mice which are previously anergic to rat Her-2-Neu.
3. The use of two subcutaneous injections of the TAA/CD40L proteins at 7 and 21 days after the subcutaneous injections of the Ad-sig-TAA/CD40L vector have been shown to boost the humoral and cellular immune response inducible by vector alone in transgenic mice previously anergic to the TAA.
4. The hMUC specific antibodies induced by the hMUC-1/ecdCD40L subcutaneous booster injections have been shown to recognize human breast cancer.

D. Reportable Outcomes.

Manuscript Published:

1. Zhang, L, Tang, Y, Linton PJ, and **Deisseroth, A.** Injection of Ad vector encoding secretable form of TAA/CD40L fusion protein induces T cell dependent immune response for against tumor cells. PNAS, 100: 15101-15106, 2003.

Manuscript in Press:

1. Tang, Y, Zhang, L, Yuan, J, Maynard, J, and **Deisseroth, A.** Vector mediated activation and tumor antigen loading of APC by CD40 ligand/tumor antigen secretory protein generates protection from cancer cell lines. Blood, in Press, 2004.

Patents:

1. Adenoviral Vector Mucin Vaccine: submitted, December, 2003.

2. Methods for Generating Immunity to Antigen: Submitted, December, 2003.

E. Conclusions.

The Ad-sig-TAA/ecdCD40L vector prime followed by the TAA/ecdCD40L protein boost vaccine can break tolerance to tumor associated antigens, can induce both a cellular and humoral response against the TAA in anergic mice, and these antibodies react with human breast cancer. This suggests that the vaccine may be of value in the prevention of recurrence of breast cancer after definitive local therapy in high risk patients.

F. References.

1. Zhang L, Tang YC., Akbulut H, Zelterman D, Linton P.J, and Deisseroth, A. An adenoviral vector cancer vaccine that delivers a tumor-associated antigen/CD40-ligand fusion protein to dendritic cells. Proc. Natl. Acad. Sci. USA, 100: 15202-15106, 2003.

2. Tang Y, Zhang L, Yuan J, Maynard J, and Deisseroth A. Vector mediated activation and tumor antigen loading of APC by CD40 ligand/tumor antigen secretory protein generates protection from cancer cell lines. Blood, In Press, 2004.

3. Gendler SJ, Lancaster CA, Taylor-Papadimitiou J, Duhig T, Peat N, Burchell J, Pemberton L, Lalain DN, and Wilson D. Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. J. Biol. Chem., 265: 15286-15293, 1990.

4. Moller H, Serttas N, Paulson H, Burchell M, and Taylor-Papadimitriou J. NMR-based determination of the binding epitope and conformational analysis of MUC-1 glycopeptides and peptides bound to the breast cancer-selective monoclonal antibody SM3. Eur. J. Biochem. 269, 1444-1455, 2002.

5. Winthrop MD, DeNardo SJ, Albrecht H, Mirick GR, Kroger LA, Lamborn KR, Venclovas C, Colvin ME, Burke PA, DeNardo GL. Selection and characterization of anti-MUC-1 scFvs intended for targeted therapy. Clin. Cancer Res. 10:3845S-53S, 2003.

6. Ren J, Agata N, Chen D, Li Y, Yu WH, Huang L, Raina D, Chen W, Lei H, Raina D, Chen W, Karbanda S, and Kufe D. Human MUC1 carcinoma-associated protein confers resistance to genotoxic anticancer agents. Cancer Cell 5: 163-175, 2004.

7. Koido S, Kashiwaba M, Chen D., Gendler S, Kufe D, and Gong JL. Journal of Immunology 165, 5713-5719, 2000.

8. Ajay P, Moniaux N, Chauhan SC, Neza JL, and Batra SK, Inhibition of MUC1 expression suppresses pancreatic tumor cell growth and metastasis. Cancer Research 64: 622-630, 2004.

9. Rowse GJ, Tempero RM, VanLith ML, Hollingsworth MA, Gendler SJ. Tolerance and immunity to MUC1 in a human MUC1 transgenic murine model. Cancer Res. 58: 315-21, 1998.

10. Xiang R, Primus FJ, Ruehlmnn JM, Niethammer AG, Silletti S, Lode HN, Dolman CS, Gillies SD, and Reisfeld RA. J. Immunol. 167, 4560-4565, 2001.

11. Taylor-Papadimitriou J, Burchell J, Miles DW, and Dalziel, M. Biochim. Biophys. Acta 1455: 301-313, 1999.

12 Rochlitz C, Figlin R, Squiban P, Salzberg M, Pless M, Herrmann R, Tartour E, Zhao Y, Bizoarne N, Baudin M, and Acres B. Phase I immunotherapy with a modified vaccinia virus expressing human MUC1 as antigen-specific immunotherapy in patients with MUC1-positive advanced cancer. The Journal of Gene Medicine, 5: 690-699, 2003.

13. Pantuck AJ, van Ophoven A, Gitlitz BJ, Tso CL, Acres B, Squiban P, Ross ME, Belldegrun AS, and Figlin RA. Phase I trial of antigen specific gene therapy using a recombinant vaccinia vius encoding MUC-1 and IL-2 in MUC-1 positive patients with advanced prostate cancer. J. Immunotherapy 27: 240-253, 2004.

14. Chen D, Xia JC, Tanaka Y, Chen HS, Koido S, Wernet O, Mukherjee P, Gendler SJ, Kufe D, and Gong JL. Immunotherapy of spontaneous mammary carcinoma with fusions of dendritic cells and mucin 1-positive carcinoma cells. Immunology 109: 300-307, 2003.

15. Mukherjee P, Madsen CS, Ginardi AR, Tinder TL, Jacobs F, Parker J, Agrawal B, Longenecker BM, and Gendler SJ. Mucin-1 specific immunotherapy in a mouse model of spontaneous breast cancer. J. Immunotherapy, 26: 47-62, 2003.

16. Musselli C, Ragupathi G, Gilewske T, Panageas KS, Spinat Y, and Livingston PO. Re-evaluation of the cellular immune response in breast cancer patients vaccinated with MUC1. Int. J. Cancer: 97: 660-667, 2002.

17. Matzinger P. Tolerance, danger and the extended family. Annual. Rev. Immunol. 12: 991-993, 1994.

18. Hodge JW, Grosenbach DWQ, Aarts WM, Poole DJ, and Schlom J. Vaccine therapy of established tumors in absence of auto-immunity. Clin. CA. Res. 9: 1837-1849, 2003.

19. Gut CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, and Muller WJ. Expression of the neuprotoconcogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc. Natl. Acad. Sci. USA 89: 10578-10582, 1992.

20. Xu T, Shu CT, Purdom E, Dang D, Ilsley D, Guo y, Weber J, Holmes SP and Lee PP. Microarray analysis reveals differences in gene expression of circulating CDE8+ T cells in melanoma patients and healthy donors. Cancer Research 64: 3661-3667, 2004.

21. Ohlen C, Kalos M, Chang LE, Shur AC, Hong DJ, Carson BD, Kokot NC, Lerner CG, Sather BD, Huseby ES, and Greenberg PD. CD * positive T cell tolerance to a tumor-associated antigen is maintained at the level of expansion rather than effector function. J. Exp. Med. 195: 1407-1418, 2002.

22. Mukherjee P, Madsen CS, Ginardi AR, Tinder TL, Jacobs F, Parker J, Agrawal B, Longenecker BM, and Gendler SJ. Mucin-1 specific immunotherapy in a mouse model of spontaneous breast cancer. J. Immunotherapy, 26: 47-62, 2003.

23. Fallaux FJ, Bout A, van der Velde I, van den Wollenberg DJ, Hehir KM, Keegan J, Auger C, Cramer SJ, van Ormondt H, van der Eb AJ, Valerio D, Hoeben RC. New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. Hum. Gene Ther. 9: 1909-1917, 1998.

24. Zhang W-W, Josephs SF, Zhou J, Fang X, Alemany R, Balague C, Dai Y, Ayares D, Prokopenko E, Lou YC, Sethi E, Hubert-Leslie, D, Ruiz, L, and Rockow-Magnone, S. Development and application of a minimal-adenoviral vector system for gene therapy of hemophilia A. Thrombosis and Haemostasis 82: 562-571, 1999.

25. Zhang W-W. Development and Application of Adenoviral Vectors for Gene Therapy of Cancer. Cancer Gene Therapy 6: 113-138, 1999.

26. LoRusso P. Phase I studies of ZD1839 in Patients with common solid tumors. Seminars in Oncology, 30: 21-29, 2003.

G. Appendices.

1. Zhang, L, Tang, Y, Linton PJ, and **Deisseroth, A.** An adenoviral vector cancer vaccine that delivers a tumor-associated antigen CD40-ligand fusion protein to dendritic cells. PNAS, 100: 15101-15106, 2003.

2. Tang, Y, Zhang, L, Yuan, J, Akbulut, H, Maynard, J, Linton, P, and **Deisseroth, A.** Multi-Step Process Through Which Adenoviral Vector Vaccine Overcomes Anergy to Tumor Associated Antigens. Blood, in Press, 2004.

An adenoviral vector cancer vaccine that delivers a tumor-associated antigen/CD40-ligand fusion protein to dendritic cells

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To develop a method to overcome the anergy that exists in tumor hosts to cancer, we have designed an adenoviral vector for the *in vivo* activation and tumor antigen loading of dendritic cells. This adenoviral vector encodes a fusion protein composed of an amino-terminal tumor-associated antigen fragment fused to the CD40 ligand (CD40L). Subcutaneous injection of an adenoviral vector encoding a fusion protein of the human papillomavirus E7 foreign antigen linked to the CD40L generates $CD8^+$ T cell-dependent immunoresistance to the growth of the E7-positive syngeneic TC-1 cancer cells in C57BL/6 mice for up to 1 year. We also studied the s.c. injection of a vector carrying the gene for the human MUC-1 (hMUC-1) self-antigen fused to the CD40L. When this vector was injected into hMUC-1.Tg mice, which are transgenic for the hMUC-1 antigen, the growth of syngeneic hMUC-1-positive LL1/LL2hMUC-1 mouse cancer cells was suppressed in 100% of the injected animals. The hMUC-1.Tg mice are anergic to the hMUC-1 antigen before the injection of the vector. These experimental results show that it is possible to use vector injection to activate a long-lasting cellular immune response against self-antigens in anergic animals. The vector-mediated *in vivo* activation, and tumor-associated antigen loading of dendritic cells does not require additional cytokine boosting to induce the immune response against the tumor cells. This vector strategy may therefore be of use in the development of immunotherapy for the many carcinomas in which the hMUC-1 antigen is overexpressed.

E7 viral antigen | MUC-1 antigen | T cells | memory cells | immunotherapy

Xiang *et al.* (1) have used an oral plasmid DNA vaccine to induce immunological resistance to the engraftment of mouse colonic carcinoma cells that are positive for the human carcinoembryonic antigen (hCEA) gene in mice transgenic for hCEA. This plasmid encodes the extracellular domain (ecd) of the hCEA linked to the ecd of the CD40 ligand (CD40L). Oral administration of this plasmid DNA vaccine carried by an attenuated strain of *Salmonella typhimurium* resulted in effective tumor-protective immunity against hCEA-positive mouse colon cancer cells. The induction of immunity in these animals was shown to involve the activation of naïve T cells and dendritic cells (DCs). This vaccine was shown to be capable of activating an immune response against hCEA in animals that were anergic to this antigen and to be 100% effective in the prophylactic setting, but this response required the use of a second treatment, IL-2, which was antibody-targeted to T cells.

To administer the tumor-associated antigen (TAA)/CD40L vaccine in a way that could affect T cells in secondary lymphoid tissue in areas of the body other than the gastrointestinal tract, and to create a therapy that does not require the antibody-targeted IL-2, we constructed replication-incompetent adenoviral vectors encoding chimeric TAA/ecdCD40L transcription units. These transcription units encode either the human papillomavirus (HPV) E7 foreign tumor antigen or the human MUC-1 (hMUC-1) self-antigen fused to the 209-aa ecd of the CD40L. This region of CD40L contains all the sequences necessary for the formation of the CD40L trimer (2). These transcription units resembled the vaccine of Xiang in that they contained a leader sequence for secretion linked to the fusion protein composed of a TAA and the CD40L.

Our vaccine differed from that of Xiang in that it used an adenoviral vector rather than a plasmid in a *Salmonella* bacterial host strain for the delivery of the TAA/ecdCD40L transcription unit. Xiang used a leucine zipper domain in the region between the hCEA antigen and the full-length CD40L, whereas we used an 8-aa linker (NDAQAPKS) between the TAA (E7 or hMUC-1) gene and the ecdCD40L gene. The Xiang construct positions the hCEA antigen at the carboxyl terminus of the full-length CD40L, whereas our vector transcription unit attached the TAA to the amino-terminal end of the ecd of the CD40L. Our arrangement should provide better binding of the CD40L to the CD40 receptor on DCs and better secretion from the cells to the extracellular space. Xiang's method required the administration of a fusion molecule composed of IL-2 linked to a T cell-targeted antibody after the hCEA/CD40L DNA vaccine, whereas our TAA/ecdCD40L adenoviral vaccine was administered without an IL-2 boost. Xiang administered his plasmid DNA vaccine orally, whereas we administered our vector vaccine s.c.

The adenoviral system used in our work has several theoretical advantages over the *Salmonella* delivery system. The expression of the TAA/CD40L gene may be at higher levels and for longer periods of time with the adenoviral delivery than with the DNA vaccine. This adenoviral TAA/ecdCD40L vector injection may thereby induce a more vigorous immune response. Although immune-specific T cells that are elicited after immunization are thought to traffic throughout the body, a propensity still exists for tissue-specific homing by memory T cells to the lymphoid sites draining the natural area of infection (3). Thus, s.c. injection of the adenoviral vector carrying the highly immunogenic TAA/ecdCD40L in the region of the tumor cells may foster the optimal trafficking of sensitized cytotoxic T cells and the generation of memory cells.

For our experiments, we chose two TAAs. The first is the E7 protein of the most commonly encountered pathogenic (cancer-causing) genotype of the HPV. We selected this antigen because it is expressed in HPV-associated intraepithelial cervical dysplasia and in the primary cells of HPV-associated cervical cancer (4). In addition, several laboratories (5, 6), including the DiMaio laboratory (7) and our own laboratory (8), have shown that the E7 protein

Abbreviations: ecd, extracellular domain; DCs, dendritic cells; sig, a signal or leader sequence for secretion to the extracellular space; HPV, human papillomavirus; CMV, cytomegalovirus; TAA, tumor-associated antigen; hCEA, human carcinoembryonic antigen; pfu, plaque-forming units.

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is required for the maintenance of the malignant phenotype of cervical cancer cells.

The second TAA is the hMUC-1 epithelial antigen. This "self-antigen" is focally expressed from birth in normal epithelial cells, but is diffusely up-regulated on epithelial surfaces in 90% of cancers of the breast, ovary, colon, and lung (9). The overexpression of hMUC-1 has been shown to promote anchorage-independent growth of tumor cells (10). Mice that have been made transgenic for hMUC-1 have been shown to develop tolerance for hMUC-1 antigen-bearing syngeneic mouse cancer cells (11).

The experimental results described in this report show that the s.c. injection of the adenoviral vector carrying the E7/ecdCD40L fusion gene generates immunological resistance to E7-positive cancer cells for at least 1 year. In addition, the s.c. injection of the adenoviral vector carrying the hMUC-1/ecdCD40L fusion gene suppresses the growth of hMUC-1-positive mouse cancer cells in hMUC-1.Tg mice that are transgenic for the hMUC-1 gene. The induction of *in vivo* resistance to the growth of the hMUC-1-positive syngeneic mouse cancer cells was shown to involve a $CD8^+$ T cell immune response against the hMUC-1 self-antigen in hMUC1.Tg transgenic mice. These mice are initially immunologically unresponsive to the hMUC-1-positive mouse cancer cells. Thus, the hMUC-1/CD40L vector injections appeared to overcome anergy. This vaccine may be of use in preventing the recurrence of epithelial malignancies after surgery and for the immunotherapy of advanced epithelial cancers that recur after surgery.

Materials and Methods

Cell Culture and Mice. The hMUC-1.Tg mice were obtained from S. Gendler (Mayo Clinic, Scottsdale, AZ) (11).

Construction of the Adenoviral Vectors. The plasmid pDC406-mCD40L was purchased from ATCC. PCR was used to produce the carboxyl-terminal 209 aa of the ecd of the mouse CD40L (ecdCD40L), which contained neither the transmembrane domain nor the cytoplasmic domain. A spacer (NDAQAPKS) was placed at the 5′ (amino-terminal) end of the transcription unit for the ecdCD40L. This fragment was inserted into the plasmid pShuttle-CMV (ref. 12; CMV, cytomegalovirus) after restriction endonuclease digestion with *Hin*dIII and *Xho*I. This vector is designated pSCMVecdCD40L.

The E7 or ecdhMUC-1 TAA fragments were inserted into the pShuttle between the CMV promoter and the linker at the amino-terminal end of the ecdCD40L transcription unit after digestion of the pSCMVecdCD40L plasmid shuttle vector with *Not*I and *Xho*I. These plasmids were designated pSCMVE7/ecdCD40L and pSCMVecdhMUC-1/ecdCD40L. In a similar fashion, we fused the GFP gene with the ecdCD40L gene and inserted it downstream of the CMV promoter in the pShuttle-CMV vector (12). This plasmid was designated pSCMVGFP/ecdCD40L. We also constructed another set of pShuttle plasmids by inserting the human growth hormone signal sequence (sig) immediately downstream of the CMV promoter in the pSCMVE7/ecdCD40L, pSCMVecdhMUC-1/ecdCD40L, and pSCMVGFP/ecdCD40L plasmids to create the pSCMVsigE7/ecdCD40L, pSCMsigecdhMUC-1/ecdCD40L, and pSCMVsigGFP/ecdCD40L shuttle plasmids. The signal sequence was placed at the amino-terminal limit of the TAA/ecdCD40L protein to promote the release of the TAA/ecdCD40L protein from the vector-infected cells. The secretion is designed to amplify the effect of the vector beyond the infected cells. In addition, for the TAA/ecdCD40L protein to activate DCs and to be taken up by the DCs, the protein must be released from the vector-infected cells.

Ad-sig-E7/ecdCD40L, Ad-sig-ecdhMUC-1/ecdCD40L, and Ad-sig-GFP/ecdCD40L are replication-incompetent adenoviral vectors that are structurally similar, except for the TAA transcription unit between the sig, which is 3′ of the CMV promoter, and at the amino-terminal end of the secretable CD40L transcription unit. All these vectors lack the E1 and E3 adenoviral genes and were assembled by homologous recombination in bacterial host strains by using the AdEasy system (12) with the pSCMVsigE7/ecdCD40L, pSCMVsigecdhMUC-1/ecdCD40L, and pSCMVsigGFP/ecdCD40L shuttle plasmids.

Sig	HPV E7	NDAQAPKS	ecdCD40 ligand (Amino acid 52-261)
	HPV E7	NDAQAPKS	wtCD40 ligand (Amino acid 1-261)
Sig	GFP	NDAQAPKS	ecdCD40 ligand (Amino acid 52-261)

Fig. 1. Organization of the adenoviral transcription units. Elements of the adenoviral vector transcription units include Sig, human growth hormone signal sequence; HPV E7, the HPV E7 gene; NDAQAPKS, a peptide linker between E7 and the ecd of the CD40L gene; ecdCD40L, amino acids 52–261 of the CD40L gene, which contain the ecd of the CD40L without the transmembrane or cytoplasmic domains; wtCD40L, amino acids 1–261 of the full-length (wild-type) CD40L gene, which include the ecd, the transmembrane domain, and the cytoplasmic domain; and the GFP gene.

Tumor Model for Ad-sig-TAA/ecdCD40L Injections. We first conducted studies to evaluate the response of tumors to the Ad-sig-E7/ecdCD40L vector injections in C57BL/6 mice. All mice were treated by using the following protocol unless otherwise described. The Ad-sig-E7/ecdCD40L adenoviral vector [1×10^8 plaque-forming units (pfu)] or control adenoviral vectors (1×10^8 pfu), as shown in Fig. 1, were injected s.c. on days 0 and 7. One week after the last s.c. vector injection, 5×10^5 TC-1 tumor cells (13) were injected s.c. at a different site. For all injections, the vector or the cells were suspended in 100 μl of PBS. Tumor growth was monitored three times each week at the injection site of the tumor cells by caliper measurement and inspection. The mice were killed when their tumors became ulcerated or reached 1.5 cm in diameter. Tumor volumes are calculated by the product of length × width^2/2. Similar experiments were carried out with the Ad-sig-ecdhMUC-1/ecdCD40L vector, the LL1/LL2hMUC-1 cell line, and the hMUC-1.Tg transgenic mouse strain.

Adoptive Transfer of Lymphocytes from Tumor-Free Mice after Injection with TC-1 Cells, and s.c. Injection of the Ad-sig-E7/ecdCD40L Vector. ***Preparation of the C57BL/6 donor mice.*** C57BL/6 mice were injected s.c. with 5×10^5 TC-1 cells. When a small tumor nodule appeared at the injection site, measurements were conducted with calipers three times a week. Five days after the injection of TC-1 cells, 1×10^8 pfu of the Ad-sig-E7/ecdCD40L vector that expressed the secretable form of the E7/ecdCD40L fusion protein were injected s.c. at a site separate from the tumor nodule in the C57BL/6 mice. This vector injection was repeated in 7 days. Several weeks later, 1×10^7 TC-1 cells were then injected s.c. in the mice that were tumor-free. Of the 10 tumor-free mice that were injected with the vector and the second dose of tumor cells, 8 mice remained tumor-free. Four of the 8 mice that remained free of tumor for 1 year were killed. The splenic T cells were isolated by negative selection by using magnetic bead separation according to StemCell Technologies (Vancouver). The purified T cells were injected i.p. into C57BL/6 *nu/nu* athymic mice that carried s.c. nodules of TC-1 tumor cells (see Fig. 4*A*).

Preparation of the recipient C57BL/6 athymic* nu/nu *mice. C57BL/6 *nu/nu* immunocompromised mice (4- to 6-week-old females) were injected s.c. with 1×10^6 TC-1 cells. One week after the injection of the TC-1 cells, the mice were injected i.p. with spleen cells from the C57BL/6 mice that had been sensitized by s.c. injection of the Ad-sig-E7/ecdCD40L vector.

***In Vivo* Depletion of $CD4^+$ or $CD8^+$ T Lymphocytes.** In a separate experiment, *in vivo* mAb ablation of CD8 (clone 2.43; ATCC TIB 210) or CD4 (clone GK1.5; ATCC TIB 207) T cell subsets was performed by i.p. injection of 0.5 mg of antibody. This antibody was

purified from the culture supernatants of hybridomas. The CD8- or CD4-depleting antibodies were injected i.p. into the immunocompetent C57BL/6 donor mice on days −5, −3, and −1 before the first vector vaccination, and every 6 days thereafter (500 μg of purified antibody per mouse per injection) during vaccination, and also on days 6, 7, 8, 10, 12, and 14 after tumor challenge (14). On day 0 and day 7, 1×10^8 pfu of the Ad-sig-E7/ecdCD40L vector were injected s.c. Seven days later, 5×10^5 E7-positive TC-1 cells were injected s.c. into each mouse. Then, the mice were observed for 3 months.

Antibody suspensions were purified from hybridoma supernatants by passage through protein G columns according to the manufacturer's instructions (Pierce). $CD4^+$ and $CD8^+$ T cell depletion was monitored by flow cytometric analysis of splenocytes isolated from test animals. On the day of tumor challenge, $CD4^+$ and $CD8^+$ cell populations were reduced by 95% and 99%, respectively.

Mice were monitored for 3 months after tumor challenge and, at that time, the tumor-free mice were killed. The spleen T cells were then isolated as described above. Five million of the $CD4^+$ T cells from the CD8-depleted, sensitized animals were mixed with five million $CD8^+$ T cells from unsensitized animals. Similarly, five million of the $CD8^+$ T cells from the CD4-depleted, sensitized animals were mixed with five million $CD4^+$ T cells from the unsensitized mice. Ten million of these mixtures of sensitized $CD8^+$ T cells and unsensitized $CD4^+$ T cells, or sensitized $CD4^+$ T cells and unsensitized $CD8^+$ T cells, were injected into C57BL/6 nu/nu mice in which one million TC-1 cells had already been injected 5 days before. The animals were then monitored for survival.

Results

Construction and Analysis of the Ad-sig-E7/ecdCD40L Vector. The organization of the transcription unit of the Ad-sig-E7/ecdCD40L vector, and several control vectors generated for analytical purposes, is shown in Fig. 1. We exposed 293 cells to the following vectors: Ad-sig-E7/ecdCD40L, Ad-sig-GFP/ecdCD40L, Ad-E7, and Ad-wtCD40L (wtCD40L contains the transmembrane and cytoplasmic domains and the ecd of the CD40L). We then subjected the protein cell lysate of the infected cells to SDS/PAGE and Western blotting. The predicted molecular weights for each of the fusion proteins encoded by the CD40L transcription units were observed on the Western blot (data not shown).

Activation of DCs by the E7/ecdCD40L Fusion Protein. To test whether the E7/ecdCD40L fusion protein could bind to the CD40 receptor and activate cytokine release from DCs, we exposed bone marrow-derived DCs (15) to the following adenoviral vectors at a multiplicity of infection of 100: Ad-sig-E7/ecdCD40L, Ad-sig-GFP/ecdCD40L, and Ad-E7. The infected DCs were then inoculated in 24-well plates at 2×10^5 cells per ml. ELISA analyses confirmed that infection of DCs by the Ad-sig-E7/ecdCD40L vector stimulated the DCs to produce 18 pg of IL-12 per 2×10^5 cells per ml in 24 h, and 88 pg of IL-12 per 2×10^5 cells per ml in 48 h. This finding was statistically significantly higher than the level of IL-12 released after exposure of the DCs to the Ad-sig-GFP/ecdCD40L vector, the Ad-E7 vectors, or the PBS control ($P < 0.0001$ as shown in Fig. 2*A*). It is possible that the level of cytokine release in cells exposed to the Ad-sig-GFP/ecdCD40L vector is lower than that seen with the Ad-sig-E7/ecdCD40L vector because of a lower level of expression of the GFP/ecdCD40L, or because of the toxicity of the GFP to cells in which it is expressed at high levels.

Similarly, as shown in Fig. 2*B*, exposure of the DCs to the Ad-sig-E7/ecdCD40L vector also induced secretion of 335 pg of IFN-γ per 2×10^5 cells per ml in 24 h and 769 pg of IFN-γ per 2×10^5 cells per ml in 48 h. This level was statistically significantly higher than the levels induced by the PBS control or the Ad-E7 or Ad-sig-GFP/ecdCD40L vectors ($P < 0.0001$). We also exposed bone marrow-derived DCs to the Ad-sig-E7/ecdCD40L vector and

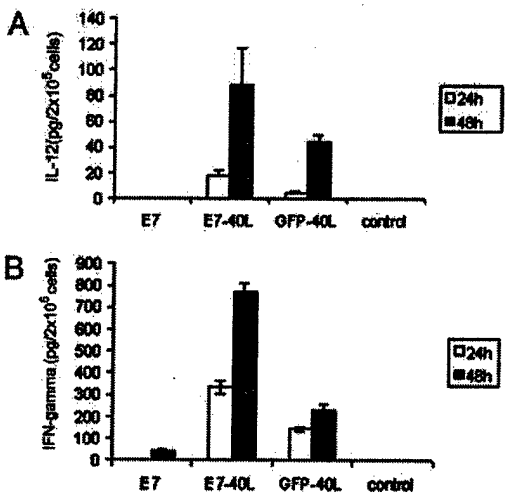

Fig. 2. Study of activation of cytokine release from DCs by exposure to the Ad-sig-E7/ecdCD40L vector. Bone marrow-derived DCs were exposed to the following vectors: E7, Ad-E7; E7–40L, Ad-sig-E7/ecdCD40L; and GFP-40L, Ad-sig-GFP/ecdCD40L. After addition of the vector at a multiplicity of infection of 100, the cells were placed in wells, and an ELISA was used to measure the production of IL-12 (*A*) and IFN-γ (*B*) by the vector-infected DCs during a 24- and a 48-h period.

showed that no IL-2 was secreted by the vector-exposed DCs (sensitivity of the assay was 0.3 pg/ml). These data suggest that the Ad-sig-E7/ecdCD40L vector can induce cytokine release from the DCs without the involvement of IL-2.

To test whether the induction of IL-12 and IFN-γ release was caused by the binding of the E7/ecdCD40L protein to the CD40 receptor on the DCs, we added 10 μg of either nonimmune IgG or 10 μg of anti-CD40L antibody to DCs that had been exposed to the Ad-sig-E7/ecdCD40L vector. Addition of this antibody reduced the IL-12 released after exposure to the vector from 27 pg per 200,000 DCs in 24 h to 0 pg per 200,000 DCs in 24 h. The IFN-γ released in the presence of the nonimmune IgG was 366 pg per 200,000 DCs in 24 h, whereas, with the addition of anti-CD40L antibody, the level released was reduced to 71 pg per 200,000 DCs in 24 h. These experiments were carried out in triplicate. These results suggest that the TAA/ecdCD40L fusion protein that is released from Ad-sig-E7/ecdCD40L-exposed DCs can assemble itself into a functional trimer that binds to the CD40 receptor on the DCs, thereby activating the DCs to release IL-12 and IFN-γ.

We were also able to show that the exposure of bone marrow-derived DCs to the Ad-sig-E7/ecdCD40L vector induces an increase in the percentage of DCs that were positive for CD80 and CD86 from 8.4% to 27.7% (for CD80) and from 3.3% to 27.4% (for CD86). Thus, the Ad-sig-E7/ecdCD40L vector induced activation of the DCs.

Subcutaneous Injection of the Ad-sig-E7/ecdCD40L Vector Confers Protection Against Engraftment and Growth of the E7-Positive TC-1 Cancer Cell Line. Female C57BL/6 mice were injected s.c. with 1×10^8 pfu of the Ad-sig-E7/ecdCD40L or Ad-sig-GFP/ecdCD40L vectors once on each of 2 days, 7 days apart. The vector-treated mice were then injected s.c. with 5×10^5 cells from the E7-positive TC-1 cancer cell line (13) 10 days after the last vector injection. As shown in Fig. 3, 0% (0 of 5) of mice injected with the Ad-sig-E7/ecdCD40L vector formed palpable tumors during 2 months of observation after a single injection of TC-1 cells, whereas 100% (5 of 5) of the mice injected with the Ad-sig-GFP/ecdCD40L vector before the injection of the TC-1 cells formed s.c. tumors, which were >500 mm^3 by 15 days after TC-1 tumor-cell injection.

The differences between the sizes of the tumors in mice injected with the Ad-sig-E7/ecdCD40L vector and the Ad-sig-GFP/

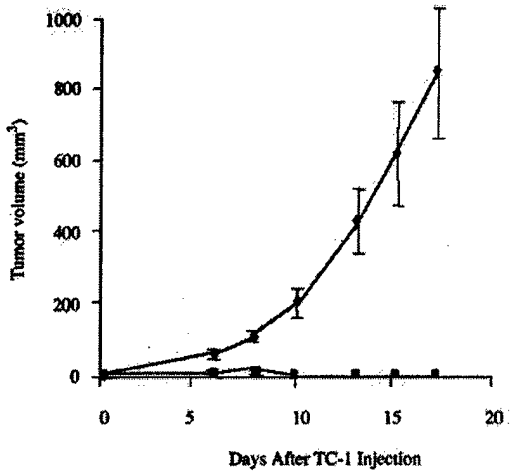

Fig. 3. Analysis of TC-1 E7-positive tumor cell line growth in mice after vector injections. Mice were injected s.c. with 1×10^8 pfu of vectors twice at 7-day intervals. Ten days later, 5×10^5 TC-1 cells were injected s.c. ($n = 5$). The following vectors were injected: ◆, Ad-sig-GFP/ecdCD40L (top line), and ■, Ad-sig-E7/ecdCD40L (bottom line). The growth of the s.c. nodule of TC-1 cells in the Ad-sig-E7/ecdCD40L-injected mice was statistically significantly different from the growth seen in the mice injected with the Ad-sig-GFP/ecdCD40L vector at the $P < 0.001$ level.

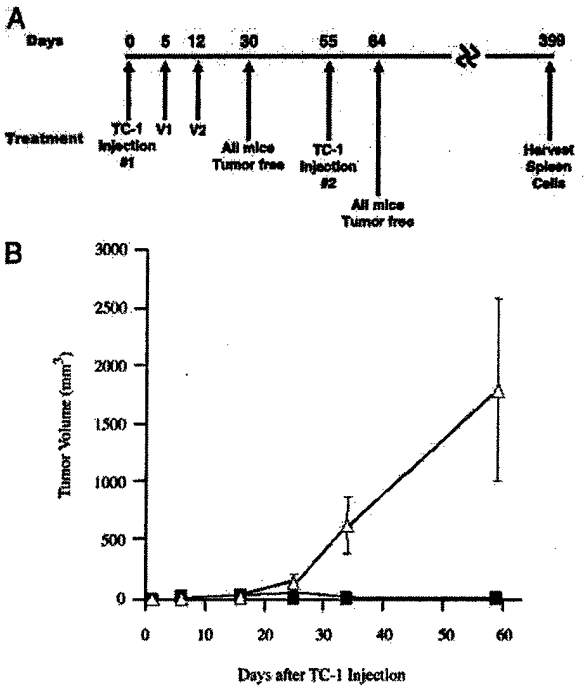

Fig. 4. (*A*) Time course of the events in the experiment presented in *B* and in Fig. 5*A*. TC-1, E7 tumor cell line; V1, first vector injection; V2, second vector injection. (*B*) Analysis of changes in the size of TC-1 E7-positive s.c. tumor nodules in C57BL/6 mice that had been growing for 5 days before injection with the Ad-sig-E7/ecdCD40L vector. As shown in *A*, 5×10^5 TC-1 cells were injected on day 0. Vectors (1×10^8 pfu) were injected on day 5 after the s.c. injection of TC-1 cells when the TC-1 tumor nodule is visible and injected again 7 days later ($n = 5$ per group). ■, Ad-sig-E7/ecdCD40L; △, PBS. The growth of the s.c. nodule of TC-1 cells in the mice injected with the Ad-sig-E7/ecdCD40L vector was statistically significantly different from that in the mice injected with PBS at the $P < 0.0001$ level.

ecdCD40L vector are statistically significantly different at the $P < 0.001$ level. The fact that the Ad-GFP/ecdCD40L vector injection does not protect the mice shows that the protective effect of the Ad-sig-E7/ecdCD40L vector, which prevents the growth of the TC-1 cell line, is not due to the adenoviral infection/transfection process.

When the tumor-free animals from the Ad-sig-E7/ecdCD40L-treated group were rechallenged with a larger dose of TC-1 cells (1×10^7 cells), a period of transient tumor growth was initially seen, which was followed by a decline in the size of the tumor nodule, ultimately leading to complete tumor regression in 100% of the five animals rechallenged with the higher dose of TC-1 cells.

Injection of the Ad-sig-E7/ecdCD40L Vector Induces Tumor Regression of Established TC-1 Tumors. Mice were first injected s.c. with 5×10^5 TC-1 cells on the hind flank. Five days later, the mice were injected s.c. at a different site with the Ad-sig-E7/ecdCD40L vector. This vector injection was repeated 7 days later. Control mice were injected s.c. with PBS 5 days after the injection of TC-1 cells (see Fig. 4*A* for time course of the experiment). As shown in Fig. 4*B*, two s.c. injections of the Ad-sig-E7/ecdCD40L vector resulted in transient growth followed by regression that was complete in 100% of the mice by 25 days after the second Ad-sig-E7/ecdCD40L vector injection. The second challenge of the immunized animals with a 20-fold increase in the number of the TC-1 tumor cells (see Fig. 4*A*) produced a brief transient growth and then complete regression of the tumor nodule in 100% of the mice injected with the Ad-sig-E7/ecdCD40L vector after the injection (data not shown). Although all the Ad-sig-E7/ecdCD40L vector-injected mice showed complete regression of tumors (see Fig. 4*B*), 100% of the animals injected with PBS developed progressive tumor growth at the TC-1 injection site within 14 days.

Splenic T Cells from Ad-sig-E7/ecdCD40L Vector-Injected C57BL/6 Mice Can Passively Transfer T Cell-Mediated Tumor Immunity to TC-1 Cells for up to 1 Year After Vector Injection. We monitored the mice from the experiment shown in Fig. 4*A* (time course of the experiment) for up to 1 year after TC-1 tumor injection, Ad-sig-E7/ecdCD40L vector injection (two injections separated by 7 days), and rechallenge with 1×10^7 TC-1 cells. Four of the eight animals that remained tumor-free for >1 year were killed and the T cells were isolated from the spleen by negative selection by using antibody and magnetic bead technology. Ten million of these splenic T cells were injected i.p. into C57BL/6 athymic nu/nu mice ($n = 7$), which had been injected s.c. 5 days previously with 5×10^5 TC-1 cells. As shown in Fig. 5*A*, the tumors in the nude mice given i.p. injections of the T cells from the Ad-sig-E7/ecdCD40L-sensitized donor mice grew into palpable s.c. nodules for 6 days and then regressed in all animals to very small tumors. The sizes of the s.c. tumor nodules were an average of 114 mm^3 on day 4, 234 mm^3 on day 6, 151 mm^3 on day 8, and 140 mm^3 on day 10 after the i.p. injection of the T cells from the sensitized immunocompetent animals shown in Fig. 4*B*. In three of the seven treated mice, the tumors regressed completely. In contrast, none of the s.c. tumors in the nude mice injected i.p. with T cells from unsensitized donor mice regressed. All the mice in the latter group died with progressive tumor growth within 3 weeks after TC-1 tumor cell injection. The mice treated with the i.p. injection of the splenic T cells from the sensitized donors were monitored for 3 months after the TC-1 challenge and remained tumor-free during that time.

A separate experiment was performed to determine the relative contribution of the $CD8^+$ vs. the $CD4^+$ T cells to the induction of immunoresistance to the TC-1 cells. The C57BL/6 mice were injected with the Ad-sig-E7/ecdCD40L vector twice, with each injection 7 days apart. Seven days after the last injection, 5×10^5 of the TC-1 cells were injected into a separate s.c. site. To deplete CD4 or CD8 T cells, the C57BL/6 donor mice were injected with antibodies against either $CD4^+$ T cells or $CD8^+$ T cells before and during the injection of the Ad-sig-E7/ecdCD40L vector (see *Materials and Methods* for the exact schedule of injections).

The efficiency of such antibody treatment for either the CD4 or

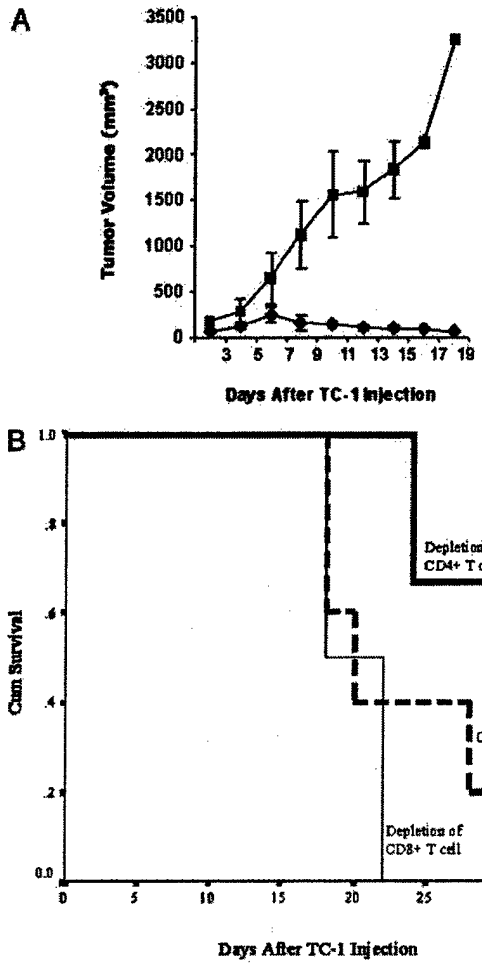

Fig. 5. (*A*) Passive transfer of immune resistance to TC-1 cell growth by using spleen cells collected from sensitized animals 1 year after vector injection. Splenic T lymphocytes were collected 1 year after Ad-sig-E7/ecdCD40L vector vaccination and challenge with TC-1 cells (see Fig. 4*A*). These sensitized spleen cells were then injected i.p. into C57BL/6 nude mice 5 days after injection of 500,000 TC-1 cells. ◆, C57BL/6 nude mice ($n = 7$) were injected s.c. with TC-1 cells and then injected i.p. 5 days later with 10×10^6 splenic lymphocytes from Ad-sig-E7/ecdCD40L-sensitized mice; ■, control animals were injected i.p. with 10×10^6 splenic T cells from unsensitized donor C57BL/6 mice 5 days after injection of TC-1 cells. (*B*) Survival of C57BL/6 nude mice after s.c. injection of TC-1 tumor cells and then i.p. injection of $CD4^+$ (thin unbroken line) or $CD8^+$ (thick unbroken line) T cell lymphocytes from Ad-sig-E7/ecdCD40L-sensitized C57BL/6 donors. Donor C57BL/6 mice were injected s.c. with the Ad-sig-E7/ecdCD40L vector at days 0 and 7. Seven days later, the mice were injected s.c. with 5×10^5 TC-1 cells. The mice were monitored for 3 months. At 5, 3, and 1 days before the vector injection, and every 6 days after the s.c. injection of the Ad-sig-E7/ecdCD40L vector, and also on days 6, 7, 8, 10, 12, and 14 after the injection of the TC-1 cells, the C57BL/6 donor mice were treated *in vivo* with antibodies specific for $CD4^+$ (thick unbroken line) or $CD8^+$ (thin unbroken line) T cell lymphocytes to deplete the respective T cell population. Then the sensitized $CD8^+$ or $CD4^+$ T cell lymphocytes from sensitized (Ad-sig-E7/ecdCD40L-injected) C57BL/6 donors were injected i.p. into C57BL/6 nude mice 7 days after s.c. injection of 5×10^5 TC-1 cells. A third group of C57BL/6 nude mice, which were control mice, did not receive passive transfer of T cells from sensitized mice (thick broken line) 7 days after s.c. injection of TC-1 cells. The mice were then monitored for cumulative survival.

CD8 depletion was determined to be 95% and 99%, respectively. Seven days after the last vector injection, the mice were injected with 5×10^5 TC-1 cells. Splenic $CD8^+$ or $CD4^+$ T cells were collected from the Ad-sig-E7/ecdCD40L vector-treated, $CD4^+$- or $CD8^+$-depleted donor mice, which had remained tumor-free for 3 months.

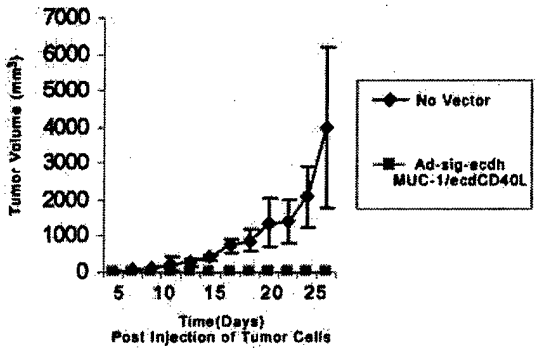

Fig. 6. The effect of the s.c. injection of the Ad-sig-ecdhMUC-1/ecdCD40L vector on the growth of the hMUC-1-positive LL1/LL2hMUC-1 cancer cell line in syngeneic hMUC-1.Tg mice. The Ad-sig-ecdhMUC-1/ecdCD40L vector was injected s.c. twice at 7-day intervals into hMUC-1.Tg mice, which were transgenic for the hMUC-1 gene. One week after the second vector injection, the mice were injected with 5×10^5 LL1/LL2hMUC-1 syngeneic mouse cancer cells that were positive for the hMUC-1 antigen. The growth of the LL1/LL2hMUC-1 cells in mice injected with the Ad-sig-ecdhMUC-1/ecdCD40L vector (■) was significantly different from the growth seen in mice not injected with vector (◆).

Five million of the $CD8^+$ T cells from CD4-depleted sensitized donors were mixed with 5 million of the splenic $CD4^+$ T cells from unsensitized donors. These cells are referred to as $CD8^+$ T cells from sensitized donors. Similarly, 5 million of the splenic $CD4^+$ T cells from CD8-depleted sensitized donors were mixed with 5 million of the splenic $CD8^+$ T cells from unsensitized donors. These cells are referred to as $CD4^+$ T cells from sensitized donors. Five million of each of these populations of cells were injected i.p. into C57BL/6 athymic nude mouse recipients in which TC-1 nodules had already been established.

As shown in Fig. 5*B*, the $CD4^+$ T cells (thin unbroken line) from the sensitized C57BL/6 mice did not protect the C57BL/6 nu/nu mice, whereas the $CD8^+$ cells from the sensitized C57BL/6 mice (thick unbroken line) prolonged the survival of the nude mice after injection of the TC-1 cells. Lymphocytes from unsensitized donor mice (broken thick line) did not protect the mice from TC-1 tumor growth. No statistically significant difference exists between the control- and CD8-depleted groups in Fig. 5 ($P = 0.21$).

Subcutaneous Injection of the Ad-sig-ecdhMUC-1/ecdCD40L Vector Overcomes Anergy for hMUC-1-Positive Cells in Mice That Are Transgenic for hMUC-1. The MUC-1 antigen is overexpressed in carcinomas of the breast, ovary, and pancreas and in other carcinomas (9). MUC-1 is also a self-antigen that is focally expressed on normal secretory epithelial cell apical surfaces. The overexpression of hMUC-1 in epithelial cancers is thought to disrupt E-cadherin function, leading to anchorage-independent growth and metastases (10). hMUC-1.Tg mice, which are transgenic for the hMUC-1 antigen, have been reported to be unresponsive immunologically to the hMUC-1 antigen (11).

We therefore s.c. injected the Ad-sig-ecdhMUC-1/ecdCD40L vector into hMUC-1.Tg mice. The hMUC-1.Tg mice had expressed the hMUC-1 antigen since birth (11). This experiment would therefore test whether the Ad-sig-ecdhMUC-1/ecdCD40L vector injection could produce resistance in anergic mice to the growth of syngeneic mouse cancer cells that were positive for the hMUC-1 antigen. As shown in Fig. 6, injection of the hMUC-1 mouse syngeneic tumor cell line, LL1/LL2hMUC-1, into the hMUC-1.Tg mice, which had not been injected with vector (◆), produced progressive growth of the LL1/LL2hMUC-1 s.c. tumor. These control animals had to be killed by 25 days after the s.c. injection of the tumor cells.

In contrast, in the hMUC-1.Tg transgenic mice that received s.c. injections of the Ad-sig-ecdhMUC-1/ecdCD40L vector, the growth

of the LL1/LL2hMUC-1 cell line was completely suppressed in all the animals tested (see ■, Fig. 6). Thus, the Ad-sig-ecdhMUC-1/ecdCD40L vector strategy can overcome anergy in 100% of the test mice without the need for additional cytokine booster treatments.

Discussion

The results of the experiments reported in this article show that the injection of the Ad-sig-E7/ecdCD40L vector into C57BL/6 mice induces T cell-mediated tumor immunity to the engraftment and growth of E7-positive tumor cells. The Ad-sig-E7/ecdCD40L also induces regression of established s.c. E7-positive tumor nodules in the C57BL/6 mice. Intraperitoneal injection of splenic T cells collected from Ad-sig-E7/ecdCD40L-vaccinated mice, which had remained tumor-free for >1 year after injection of the Ad-sig-E7/ecdCD40L vector and tumor challenge, induced regressions of TC-1 tumors already growing in immunocompromised athymic nude recipient mice (see Fig. 5*A*). This experiment and the experimental results summarized in Fig. 5*B* show that the effect of the Ad-sig-E7/ecdCD40L injections on E7-positive TC-1 cells is mediated by a $CD8^+$ T cell-dependent immune response that lasts for >1 year.

The study of the effect of s.c. injection of the Ad-sig-ecdhMUC-1/ecdCD40L vector into hMUC-1.Tg mice (11) allowed us to test whether the Ad-sig-ecdhMUC-1/ecdCD40L vector injection by itself could activate a $CD8^+$ T cell immune response against the hMUC-1-positive mouse cells in 100% of the animals otherwise anergic to the hMUC-1 antigen. This proved to be the case.

The Ad-sig-TAA/ecdCD40L vector strategy described in this article is unique in several ways. It has been shown to overcome anergy in a transgenic mouse model in 100% of the test mice without the use of cytokine boosting. In addition, it can generate cellular immunity for up to a year, which indicates that the vector strategy outlined in this article induces memory cells.

The Garen laboratory (16) has recently reported that the s.c. injection of 293 cells infected with an adenoviral vector carrying an E7/IgGFc transcription unit can suppress the growth of the TC-1 cell line in a syngeneic mouse model. An interesting parallel exists between the Ad-sig-E7/IgGFc vector of Garen and the Ad-sig-TAA/ecdCD40L vector described in this article: an *in vivo* continuous release strategy is used in both sets of experiments to generate an immune response against a foreign antigen.

The finding about the Ad-TAA/ecdCD40L vector that is different from the findings reported by Garen and his colleagues is the successful activation of an immune response against self-antigens without the need for cytokine booster treatments. The successful induction of T cell-mediated tumor immunity in 100% of the test anergic animals with the Ad-sig-ecdhMUC-1/ecdCD40L vector shows that the adenoviral vector delivery vehicle is superior to the oral DNA vaccine of Xiang (1) that was delivered in *Salmonella*. No need exists to use additional IL-2 treatments after the TAA-ecdCD40L vaccination in the case of the Ad-sig-ecdhMUC-1/ecdCD40L vector, whereas the IL-2 treatments are required to induce an antitumor immune response in 100% of the anergic animals with the *Salmonella* DNA vaccine approach. This experimental result confirms the superiority of the adenoviral vector approach.

The introduction of the secretory sequence at the amino-terminal end of the TAA/ecdCD40L transcription unit and the deletion of the transmembrane domain of the CD40L ensure that this protein will be secreted from the infected cells. Previous reports of the use (17) of Ad-CD40L vectors did not use a secretable CD40L transcription unit because the goal was to display the CD40L on the plasma membrane of the DCs. In this work (17), the effect of the vector was limited to the vector-infected cells and the cells that they directly stimulate. The use of the secretable vector in our work produces an amplification effect beyond the vector infected cells to uninfected DCs.

The statistically significant increase in secretion of IL-12 and IFN-γ at 48 h after exposure of the DCs to the Ad-sig-E7/ecdCD40L vector, as compared with the PBS control (see Fig. 2), shows that the E7/ecdCD40L fusion protein can bind to the CD40 receptor on DCs and stimulate the CD40 receptor sufficiently well to activate the DCs. The fact that the IL-12 and IFN-γ secretion after exposure to the Ad-sig-E7/ecdCD40L vector is statistically significantly greater than the secretion after exposure of the DCs to the Ad-E7 or Ad-sig-GFP/ecdCD40L vectors (see Fig. 2) shows that the increased secretion is not due to the effect of the adenoviral infection/transfection process on the DCs.

One possible complication of inducing an immune response against a self-antigen associated with cancer is that this could generate an autoimmune disease against the normal tissues that normally express that antigen. Several considerations suggest that this will not be a problem with the MUC-1 antigen. First, although the MUC-1 antigen is overexpressed diffusely at very high levels throughout neoplastic epithelial cells, MUC-1 is expressed only very focally and at very low levels in normal epithelial apical structures. Although experiments using a tandem-repeat protein of the MUC-1 antigen with a *Leishmania*-derived protein as adjuvant generated in chimpanzees $CD4^+$ helper and $CD8^+$ cytotoxic responses, no signs of autoimmune disease were detected for up to 1 year after the administration of the vaccine (18). Passive transfer of anti-MUC-12 antibodies does not cause autoimmune disease (19).

The results obtained with the Ad-sig-E7/ecdCD40L vector injections suggest that these vectors may be useful in the prevention of HPV-associated cervical cancer and for the treatment of metastatic cervical cancer. In addition, because hMUC-1 is a self-antigen that is overexpressed in 90% of carcinomas of the breast, ovary, colon, and lung, it is possible that the Ad-sig-ecdhMUC-1/ecdCD40L vector vaccine strategy described in this report could be of use in activating an immune response against a wide range of epithelial neoplasms in human patients.

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1. Xiang, R., Primus, F. J., Ruehlmnn, J. M., Niethammer, A. G., Silletti, S., Lode, H. N., Dolman, C. S., Gillies, S. D. & Reisfeld, R. A. (2001) *J. Immunol.* **167,** 4560–4565.
2. Karpuses, M., Hsu, Y. M., Wang, J. H., Thompson, J., Lederman, S., Chess, L. & Thomas, D. (1995) *Structure (London)* **3,** 1031–1039.
3. Gallichan, W. S. & Rosenthal, K. L. (1996) *J. Exp. Med.* **184,** 1879–1890.
4. Bosch, F. X., Manos, M. M., Munoz, N., Sherman, M., Jansen, A. M., Peto, J., Schiffman, M. H., Moreno, V., Kurman, R. & Shah, K. V. (1995) *J. Natl. Cancer Inst.* **87,** 796–802.
5. Morozov, A., Shiyanov, P., Barr, E., Leiden, J. M. & Raychaudhuri, P. (1997) *J. Virol.* **71,** 3451–3457.
6. Kadish, A. S., Timmons, P., Wang, Y., Ho, G. Y. F., Burk, R. D., Ketz, J., He, W., Romney, S. L., Johnson, A., Angeletti, R., *et al.* (2002) *Cancer Epidemiol. Biomarkers Prev.* **11,** 483–488.
7. Goodwin, E. C. & DiMaio, D. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 12513–12517.
8. Hu, G., Liu, W., Mendelsohn, J., Ellis, L., Radinsky, R. & Deisseroth, A. B. (1997) *J. Natl. Cancer Inst.* **89,** 1271–1277.
9. Taylor-Papadimitriou, J., Burchell, J., Miles, D. W. & Dalziel, M. (1999) *Biochim. Biophys. Acta* **1455,** 301–313.
10. Koido, S., Kashiwaba, M., Chen, D. S., Gendler, S., Kufe, D. & Gong, J. L. (2000) *J. Immunol.* **165,** 5713–5719.
11. Tempero, R. M., VanLith, M. L., Morikane, K., Rowse, G. J., Gendler, S. J. & Hollingsworth, M. A. (1998) *J. Immunol.* **161,** 5500–5506.
12. He, Z., Wlazlo, A. P., Kowalczyk, D., Cheng, J., Xiang, Z. Q., Giles-Davis, W. & Ertl, H. C. J. (2000) *Virology* **270,** 146–161.
13. Lin, K. Y., Guarnieri, F. G., Staveley-O'Carroll, K. F., Levitsky, H. I., August, J. T., Pardoll, D. M. & Wu, T. C. (1996) *Cancer Res.* **56,** 21–26.
14. Ribas, A., Butterfield, L. H., Amarnani, S. N., Dissette, V. B., Kim, D., Meng, W. S., Miranda, G. A., Wang, H. J., McBride, W. H., Glaspy, J. A. & Economou, J. S. (2001) *Cancer Res.* **61,** 8787–8793.
15. Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S. & Steinman, R. M. (1992) *J. Exp. Med.* **176,** 1693–1702.
16. Li, J., Sun, Y. & Garen, A. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 16232–16236.
17. Kikuchi, T., Moore, M. A. & Crystal, R. G. (2000) *Blood* **96,** 91–99.
18. Barratt-Boyes, S. M., Vlad, A. & Finn, O. J. (1999) *Clin. Cancer Res.* **5,** 1918–1924.
19. Tempero, R. M., Rowe, G. J., Gendler, S. J. & Hollingsworth, M. A. (1999) *Int. J. Cancer* **80,** 595–599.

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Appendix 2

Multi-Step Process Through Which Adenoviral Vector Vaccine Overcomes Anergy to Tumor Associated Antigens

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Abstract

We previously reported (1) that the subcutaneous injection of the Ad-sig-TAA/ecdCD40L) vector can overcome the anergy in tumor hosts against TAA. Our goal in the present work was to characterize the multiple steps that are involved in overcoming the anergy which exists in tumor hosts to TAA. Our studies showed that the subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector resulted in the secretion of the TAA/ecdCD40L protein for at least 10 days from the infected cells. The binding of the TAA/ecdCD40L protein to DCs resulted in induction of expression of the CCR-7 chemokine receptor and cytokine release. This was followed by migration of the DCs to regional lymph nodes. Tetramer staining, ELISPOT assay, and cytotoxicity assays all showed that the Ad-sig-TAA/ecdCD40L vector increased the levels of splenic CD8+ T cells specific for the two TAA (human MUC1 and HPV E7) tested. Vaccination with the Ad-sig-hMUC1/ecdCD40L vector suppressed the growth of human MUC1 (hMUC1) antigen positive tumor cells in 100% of the test mice which were previously anergic to the hMUC1 antigen. These data suggest that the Ad-sig-TAA-ecd/ecdCD40L vector injections may be of value in the treatment of the many epithelial malignancies in which TAA like hMUC1 are over expressed.

Introduction

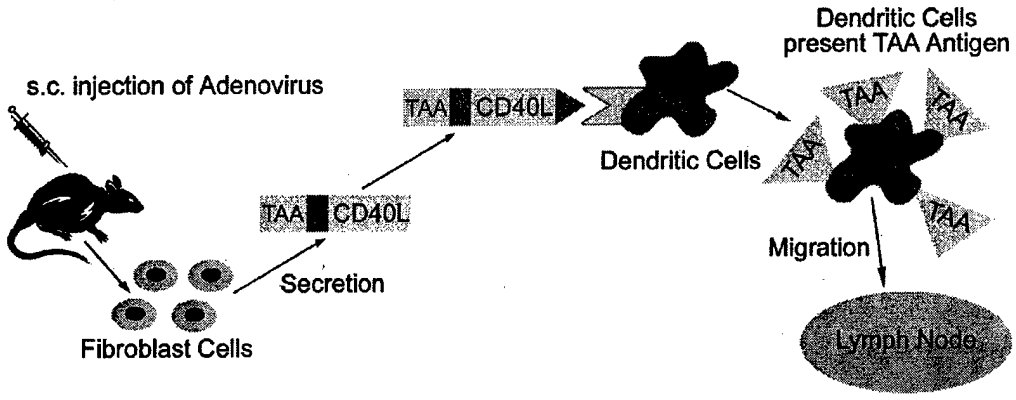
Dendritic cells (DC) are specialized cells of the immune system responsible for the initiation and regulation of both cellular and humoral responses. The ability of DCs to regulate immunity is dependent on DC maturation. In the absence of co-stimulatory molecule expression on the surface of the DC, presentation of the TAA to naïve T cells can lead to anergy of T cells due to the induction of apoptosis in the T cells (2).

Human dendritic cells require multiple activation signals for the efficient generation of tumor antigen-specific T lymphocytes (3-4). These changes endow DCs with the ability to co-stimulate antigen-specific $CD8^+$ and $CD4^+$ T cell responses, and to foster $CD8^+$ T cell differentiation into cytotoxic lymphocytes (CTL) (5-6). The fact that antigen-loaded DCs can generate an anti-tumor immune response capable of eradicating established tumors in vivo has been documented in a number of animal tumor models. Strategies for loading DCs with TAA include: the pulsing of tumor cell RNA into DCs, the mixing of tumor cell lysates with DCs, and the in vitro addition of recombinant peptides of proven binding capability to DCs (7-13). Dendritic-cell vaccination leads to tumor regression in selected advanced cancer patients but the weight of clinical trial data suggest that in vivo activation and tumor antigen loading of dendritic cells might provide an advantage over in vitro activation strategies.

In order to develop an in vitro strategy of activation and tumor antigen loading of DCs with which to overcome anergy to TAA, we built upon the oral DNA vaccine/IL2 targeting strategy of Xiang et al (14) to create an adenoviral vector (Ad-sig-TAA/ecdCD40L) vaccine. The Ad-sig-TAA/ecdCD40L adenoviral vector encodes a secretable (sig) form of a TAA fused to the extra cellular domain (ecd) of the CD40 ligand (CD40L). The ecd of the CD40L contains all of the sequences necessary to form a functional trimeric CD40L (15). Our previous studies with this vector shows that the subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector induced immune resistance to the growth of TAA positive cancer cells for over one year (1).

In the present work, we sought to characterize the multiple steps through which the Ad-sig-TAA/ecdCD40L vector induces an immune response to TAA in anergic animals. As shown in Figure 1A, this involves secretion of the TAA/ecdCD40L protein from the Ad-sig-TAA/ecdCD40L vector infected cells near the subcutaneous injection site for over 10 days. The binding of the TAA/ecdCD40L protein to the DCs resulted in the activation of cytokine release, increased levels of the CCR-7 chemokine, and increased membrane levels of the CD80 and CD86 receptors as well. This induced migration of the DCs, which were displaying the TAA peptides on their surface class I MHC molecules, resulted in increases in the number of TAA specific CD8+ T cells which are competent to recognize and kill cancer cells bearing the TAA (7, 16).

Fig 1A



We studied two types of TAA in this vector vaccination strategy: the human papilloma virus (HPV) E7 foreign antigen which has been shown to be a strong stimulus of the cellular immune response (17-20), and the ecd of the human Mucin-1 (hMUC1) self antigen, which is expressed focally at low levels on normal epithelial cellular surfaces (21-24). The MUC1 antigen is expressed at high levels diffusely in neoplastic epithelial mucosal cells thereby disrupting the regulation of anchorage dependent growth, which leads to metastases (22-23). The MUC1 antigen is a self-protein, which is over expressed in carcinomas of the breast, ovary, lung, prostate, colon, and pancreas as well as other carcinomas (21). The overexpression in epithelial cancers is thought to disrupt E-cadherin function leading to anchorage independent growth and metastases (22). Although non-MHC-restricted cytotoxic T cell responses to MUC1 have been reported in patients with breast cancer (23), human MUC1 transgenic mice (MUC1.Tg) have been reported to be unresponsive to stimulation with human MUC1 antigen (24).

Our results show that the immunization of hMUC1 transgenic mice, which are anergic to the hMUC1 antigen (24), with the Ad-sig-hMUC1/ecdCD40L vector, induces a CD8+ T cell dependent systemic Th1 immune response which is antigen specific, HLA restricted, and overcomes the block in proliferation which exists in T cells in anergic hosts. The vaccination increases the frequency of hMUC1 specific T cells in the spleens of injected mice. This response requires the Ad-sig-ecdhMUC1/ecdCD40L adenoviral vector and cannot be produced by subcutaneous injection of the hMUC1/ecdCD40L protein alone. Using a similar vector system, but with the E7 antigen in place of the hMUC1 antigen, we showed that the Ad-sig-E7/ecdCD40L vector injection induces an immune response against E7 positive TC-1 tumor cells in 100% of the injected mice for up to a year. These results suggest that Ad-sig-TAA/ecdCD40L vector injections induce a memory cell response against TAA positive tumor cells without the need of additional cytokine boosting treatments.

Methods

Mice and Cell Lines. Six- to 8-wk-old C57BL/6 mice were purchased from Harlan. MUC1 transgenic mice-C57/BL6 /human MUC1 (24) were obtained from Dr. S. Gendler of Mayo Clinic Scottsdale, and then bred on site.

Construction of Recombinant Adenoviruses. The E7/ecdCD40L fusion gene was constructed by ligating the amino terminal end of the extra cellular domain of CD40L to an octapeptide linker (NDAQAPKS) which was linked in turn to the carboxyl terminal end of a TAA, the amino terminal end of which was linked to a secretory signal sequence. The oligonucleotide for E7 was: 5'-TGG GTT CCA GGT TCC ACT GGT GAC ATG CAT GGA G AT ACA CCT AC-3' and 5'-CCG CTC GAG TGG TTT CTG AGA ACA GAT GGG GCA C -3'. This oligonucleotide was cloned into the pcDNA3TOPO vector. The coding sequences for the full length mouse CD40 ligand were generated by using the following primers: 5'-GAGAC CTC GAG AAC GAC GCA CAA GCA CCA AAA AGC ATG ATA GAA ACA TAC AGC CAA C-3' and 5'-CCG CGC CCC AAG CTT ATC AGA GTT TGA GTA AGC CAA AAG-3'. The CD40L template is the plasmid pDC406-mCD40L (American Type Culture Collection). The PCR conditions are as per protocol from Tgo DNA polymerase kit (Roche Diagnostics Gmbh): 94°C for 3 min, 25 cycles at 94°C for 30 seconds, 56°C for 45 seconds,72°C for 45 seconds, and 1 cycle for 72°C for 7 minutes. The PCR fragment was inserted into the

plasmid pcDNA3-E7 after restriction endonuclease digestion with XbaI (TCTAGA) and Xho I (CTCGAG). This vector was named pCDNA3 "CE7 /wtCD40L. The E7/wt encoding DNA was cut from pCDNA3CE7/wtCD40L using HindIII-XbaI restriction endonuclease digestion which was then inserted into pShuttle-CMV downstream of the CMV promoter. This plasmid is designated pShuttle- E7 /wtCD40L .

The ecdCD40L fragment for pShuttle-ecdCD40L was generated by PCR encoding the mouse IgG kappa chain by four rounds of PCR amplification (1st round: primers 1 +5; 2nd round: primer 2+5; 3rd round: primer 3+5; 4th round: primer 4+5). The primers are as follows: 1. 5'- CTG CTCTGG GTT CCA GGT TCC ACT GGT GAC AAG GTC GAA GAG GAA GTA AAC C-3'; 2. 5'-TG CTC TGG GTT CCA GGT TCC ACT GGT GAC ATG CAT G-3'; 3. 5'-TC CTG CTA TGG GTA CTG CTG CTC TGG GTT CCA GGT TC3'; 4. 5'-ACG ATG GAG ACA GAC ACA C TC CTG CTA TGG GTA CTG CTG-3'; 5. 5'-CCG CGC CCC TCT AGA ATC AGA GTT TGA GTA AGC CAA AAG-3'.

The CD40L template is the Plasmid pDC406-mCD40L (American Type Culture Collection). The PCR conditions are per protocol from Tgo DNA polymerase kit (Roche Diagnostics: conditions are the same as above. The fragments of the ecdCD40L were cloned into the pcDNA3.1TOPO vector (Invitrogen, San Diego, CA), then cut from the pCDNA3 -hMUC1/ecdCD40L vector using HindIII-XbaI restriction endonuclease digestion and inserted into pShuttle-CMV downstream of the CMV promoter and named as pShuttle-ecdCD40L.

A transcription unit that included DNA encoding the signal sequence of the mouse IgG kappa chain gene upstream of DNA encoding human MUC-1 was generated by PCR using plasmid pcDNA3-hMUC-1 (gift of Finn O.J., University of Pittsburgh School of Medicine) and the following primers: DNA encoding the mouse IgG kappa chain METDTLLLWVLLLWVPGSTGD (single letter amino acid code) was prepared by PCR amplification to generate the full 21 amino acid mouse IgG kappa chain signal sequence.1. 5'-CCACC ATG GAG ACA GAC ACA CTC CTG CTA TGG GTA CTG CTG-3'; 2. 5'- TC CTG CTA TGG GTA CTG CTG CTC TGG GTT CCA GGT TC-3'; 3. 5'- TG CTC TGG GTT CCA GGT TCC ACT GGT GAC GAT G -3'; 4. 5'- GGT TCC ACT GGT GAC GAT GTC ACC TCG GTC CCA GTC-3'; 5. 5'- GAG CTC GAG ATT GTG GAC TGG AGG GGC GGT G-3'. K/hMUC-1 with the upstream kappa signal sequence was generated by four rounds of PCR amplification (1st round: primers 4 +5; 2nd round: primer 3+5; 3rd round: primer 2+5; 4th round: primer 1+5). The PCR conditions are the same as above. The hMUC-1 encoding DNA was cloned into the pcDNA3.1TOPO vector (Invitrogen, San Diego, CA) forming pcDNA-hMUC-1. A pair of PCR primers was designed for ecdCD40L without the cytoplasmic and transmembrane domains: 5'-CCG CTC GAG AAC GAC GCA CAA GCA CCA AAA TCA AAG GTC GAA GAG GAA GTA -3'; 5'-GCG GGC CCG CGG CCG CCG CTA GTC TAG AGA GTT TGA GTA AGC CAA AAG ATG AG-3'. The CD40L template is the plasmid pDC406-mCD40L (American Type Culture Collection). The PCR conditions are as per protocol from Tgo DNA polymerase kit (Roche Diagnostics Gmbh) which are the same as above. The PCR fragment was inserted into the plasmid pcDNA-hMUC-1 after

restriction endonuclease digestion with XbaI (TCTAGA) and Xho I (CTCGAG). This vector was named as pCDNA3-hMUC1/ecdCD40L. The hMUC1/ecdCD40L encoding DNA was cut from the pCDNA3 -hMUC1 /ecdCD40L vector using HindIII-XbaI restriction endonuclease digestion and inserted into pShuttle-CMV downstream of the CMV promoter. The plasmid is designated pShuttle-hMUC1/ecdCD40L.

The coding sequences for the full length mouse CD40L were generated by using the following primers: 5'-GAG ACC TCG AGA ACG ACG CAC AAG CAC CAA AAA GCA TGA TAG AAA CAT ACA GCC AAC-3' and 5'-CCG CGC CCC AAG CTT ATC AGA GTT TGA GTA AGC CAA AAG-3'. The CD40L template is the Plasmid pDC4mCD40L (American Type Culture Collection). The PCR conditions are as protocol from Tgo DNA polymerase kit (Roche Diagnostics Gmbh) are the same as above. Using PCR methods, in some vectors, we added the mouse HSF1 trimer domain between MUC-1 and CD40L and a His tag at the end of the CD40L. The fragments of the TAA/CD40L fusion were inserted downstream of the CMV promoter in the pShuttleCMV expression vector using the XhoI and XbaI restriction sites. The ecd of the CD40L and the full length-wtCD40L was amplified by PCR primers and cloned into the pShuttleCMV plasmid using the Hind III and Xba I restriction endonuclease sites. The recombinant adenoviral vectors were generated using the AdEasy vector system (25).

All populations of vector particles used in the experiments described in this paper were shown to contain less than five replication competent adenoviral particles (RCAs)/ 1 X 10^{10} viral particles (VP).

Western Blotting and In vitro Expression of the E7/ecdCD40L Transcription Unit. Both Western blotting and in vitro cell free transcription/translation were used to analyze protein expression from the vector transcription units. The coupled in vitro transcription-translation system of RRL (TNT kits from Promega Corp.) was used for the synthesis of the protein products of the transgenes of the following vectors: Ad-sig-E7/ecdCD40L, Ad-E7/wtCD40L (where wt=the full length or wild type CD40L gene), Ad-sig-ecdCD40L, Ad-wtCD40L and Ad-sig-ecdhMUC1/ecdCD40L. The protein cell lysate derived from 293 cells infected by each adenoviral vector described above at MOI 40 was fractionated on a 10% reducing SDS-PAGE gel and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). After blocking with 5% nonfat milk for 2 hours at room temperature, the membrane was probed with an antibody against the specific mouse CD40L (mCD40LM eBioscience) in TBS-T buffer (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, and 0.5% Tween 20) in the presence of 2% BSA overnight. After washing four times with TBS-T buffer, the blot was incubated with goat anti-hamster alkaline phosphatase conjugated antibody (Jackson Immunoresearch) for 1 hour. The immunoreactive bands were visualized on membrane by using the ProtoBlot II AP system (Promega Corp.).

Assay for Binding of the TAA/CD40L Protein to DCs. The DCs were derived from incubation of bone marrow mononuclear cells in GMCSF and IL4 for 7 days, followed by purification to a purity of 78% dendritic cells. The TAA/CD40L proteins were generated by exposing 293 cells to either the Ad-sig-E7/ecdCD40L vector (Figure 1D,

Panels A and B) or the Ad-sig-ecdhMUC-1/ecdCD40L(HIS tagged) vector (Figure 1D, Panel C). In Panels A and B, no purification of the proteins was carried out, whereas in Panel C, Nickel column purification of the ecdhMUC-1/ecdCD40L proteins was carried out. The TAA/CD40L proteins were fluorescently labeled with the Fluoreporter FITC protein labeling kit (Molecular Probes), were added to the DCs at a final concentration of 10 μg/m and incubated for 30 min. The cells were then washed three times with cold medium and fixed with 1% Para formaldehyde and observed in a fluorescence microscope.

Assay for Activation of Bone-Marrow Derived DCs. The DCs were incubated with the supernatant from 293 cells infected by Ad-sig-TAA/ecdCD40L adenoviral vectors, and then plated in 24-well plates at 2×10^5 cells/ml. After incubation for 24 hours and 48 hours at 37°C, the supernatant fluid (1ml) was harvested and centrifuged to remove debris. The level of murine IL-12 or IFN-gamma released into the culture medium from vector-infected cells was assessed by enzyme-linked immunoadsorbent assay (ELISA), using the mouse IL-12 p70 or IFN-gamma R & D Systems respectively. Bone marrow cells were incubated for 5 days in GMCSF and IL-. The DC's were purified with the SpinSep™ Mouse Dendritic Cell enrichment kit. The forward and side scatter analysis of the populations before and after fractionation are given below in Figures 1B and 1C. We then stained the bone marrow derived DC before and after fractionation with PE-labeled CD11c antibody, incubating non-enriched and enriched cells for 10 minutes on ice with 5% normal rat serum to block non-specific background prior to addition of fluorochrome-

conjugated antibodies. Then we stained the DC fractions with PE-labeled CD11c antibody.

Detection of CCR-7 mRNA by RT-PCR. Total RNA extracted from DCs was analyzed for CCR-7 mRNA as described previously (26). The primers for detection of CCR7 and GAPDH control were as follows: for CCR7 Sense: 5'-TCC TCC TAA TTC TTC CCT TC-3'; for CCR7 Antisense: 5'-AAA CTC ATA GCC AGC ATA GG-3'); for GAPDH Sense: 5'-TTG TGA TGG GTG AAC CAC-3'; and for GAPDH Antisense: 5'-CCA TGT AGG CCA TGA AGT CC-3'. The expected size of the amplified fragments was 400bp for CCR7 and 525bp for GAPDH. Amplified samples were resolved on ethidium bromide-stained agarose gels. Total cellular RNA was extracted using the Trizol reagent (Life Technologies, Burlington, Ontario, Canada). The RT-PCR was performed on 5ug of RNA for the reverse transcription reaction. One half of each cDNA product was used to amplify the CCR-7 and GAPDH.

DC Migration Assays. Bone marrow derived DCs were loaded with the CFDA SE supravital dye for 15 min at 37°C. The rinsed DCs were mixed with each recombinant adenoviral vector at MOI 200 and injected into the left flank of the test mouse. Three days later, the axillary lymph nodes draining the region of the injection site for the DCs were removed, frozen tissue sections made and then observed in the fluoresence microscope.

Immunohistochemical Staining. Vaccinated mice were sacrificed 3 and 10 days after injection of the Ad-sig-E7/ecdCD40 vector. The skin at the site of subcutaneous vector injection was biopsied, embedded in OCT solution, and cut into 5-μm sections. The slides were incubated with rat anti-CD40L antibody (Bioscience), exposed to biotinylated goat anti-rat IgG antibody (1:200 dilution) and avidin-biotin complex (Burlingame, CA). The stained slides were then mounted, and studied under a fluorescence microscope.

Tetramer and ELISPOT Assays. PE-labeled H-2D^b tetramers containing HPV16 $E7_{49-57}$ peptide (RAHYNIVTF) were purchased from Beckman Coulter and used for the FACS analysis of peptide specific CTL immunity. Tetramer positive and $CD8^+$ cells are shown as a percentage of total spleen cells. The presence of E7- and hMUC1 specific effector T cells in the immunized mice was also assessed by carrying out ELISPOT assays, as previously described (27).

Cytotoxicity Assay. 5 X 10^3 E7 positive TC-1 target cells or LL2/LL1hMUC1 positive target cells were incubated with splenic mononuclear cells (effector cells) at varying effector/target ratios (100/1,20/1 and 5/1) for 4 hours at 37°C, in culture media containing 5% FBS. The effector cells had been pre-stimulated with the TAA positive cancer cells for 5 days in vitro before the in vitro cytotoxicity assay. Cell–mediated cytotoxicity was determined using a nonradioactive LDH release assay. Student's unpaired t-test was used to determine the differences among the various groups in cytotoxicity assays. Statistical significance was defined by the 0.01 level.

In Vivo Efficacy Experiment in Mouse Model. Mice (5 or 10 per group) were vaccinated via subcutaneous injection with 1 x 10^8 PFU of the Ad-sig-TAA/ecdCD40L,

Ad-TAA, Ad-TAA/wtCD40L, Ad-sig-CD40L, Ad-wtCD40L or Ad-sig-ecdhMUC1/ecdCD40L vectors. One week later, mice were boosted with the same adenoviral vector regimen as the first vaccination. One week after the last vaccination, mice were challenged by subcutaneous injection of 5 x 10^5 TAA positive cancer cells. Tumor volumes were measured in centimeters by caliper, and the volume was calculated as tumor volume=length x (width^2)/2 (this assumes an elliptical shape).

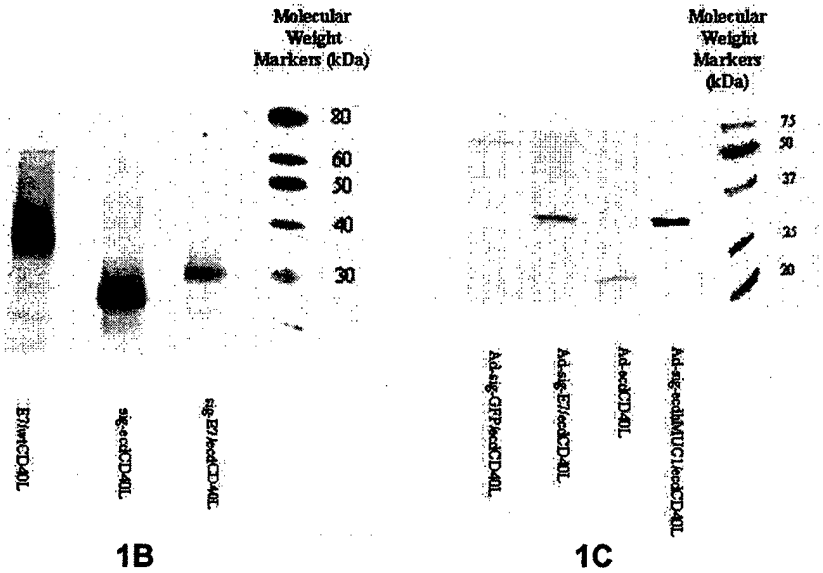
Analysis of p44/42 MAP Kinase and SAPK/JNK Phosphorylation. Western blot analysis of p44/42 and SAPK/JNK was carried out with kits (#9100 for P44/42 and #9250 for SAPK/JNK) from New England Biolabs. Responder splenocytes were isolated from vaccinated mice and enriched in $CD8^+$ cells using a murine CD8 T cell enrichment kit (Stemcell Tech catalogue #13033). Bone marrow derived dendritic cell were infected with Ad-sig-ecdlMUC1/ecdCD40L for 2 hours, then washed with phosphate buffered saline, and incubated for 48 hours (28. The responder cells were mixed in a 1:1 ratio with Ad-sig-ecdhMUC1/ecdCD40L infected APC, and Western blot analysis was carried out at the indicated time points.

Statistics. All parameters were analyzed using Student's t test, or ANOVA followed by Scheffé's procedure for multiple comparisons as post-hoc analysis; all data shown is presented as mean ± S.E. of the mean (S.E).

Results

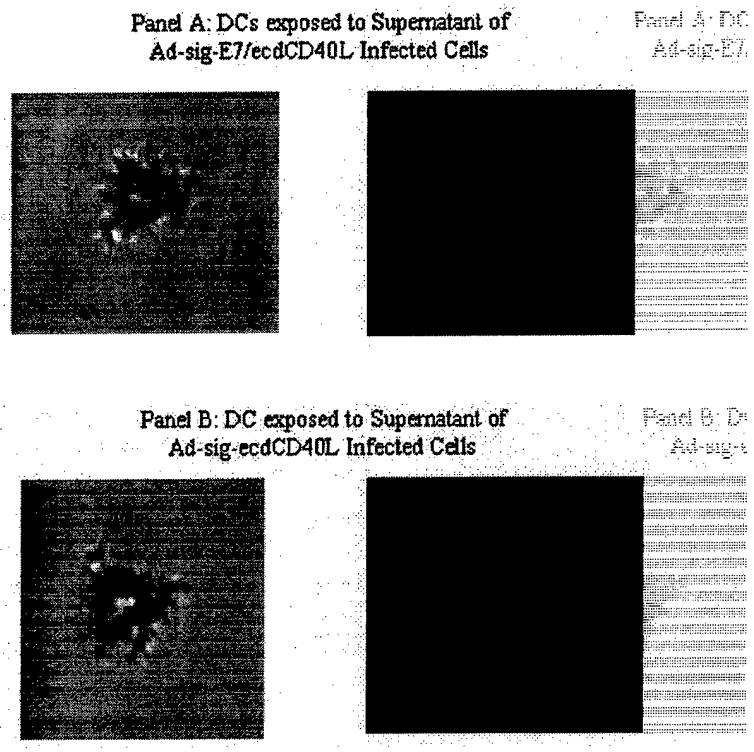
The TAA/ecdCD40L Protein Binds to DCs. Cell free coupled transcription/translation and western blot analysis of the E7/ecdCD40L, E7, ecdCD40L, E7/wtCD40L and wtCD40L proteins were used to study the molecular weights of the proteins produced in cells infected by the Ad-sig-E7/ecdCD40L, Ad-sig-E7, Ad-sig-ecdCD40L, Ad-E7/wtCD40L and Ad-wtCD40L vectors respectively. As shown in Figures 1B and 1C, the molecular weights of these proteins are those predicted.

Fig 1B and Fig 1C



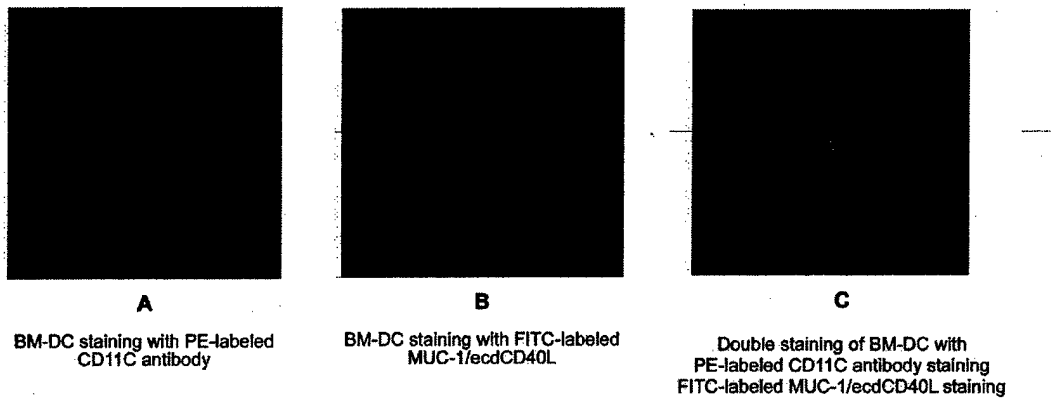
We then collected the TAA/ecdCD40L proteins from vector infected 293 cells and labeled these proteins with fluorescein (see methods). These proteins were then incubated in vitro with bone marrow derived DCs (fractionated to 78% purity) for 30 min at 4^0C. The DCs were washed and photographed once with light microscopy and again with fluorescent microscopy. As shown in Panels A and B of Figure 1D, the secretable form of the E7/ecdCD40L can bind to the DCs.

Fig 1D: Panels A and B



A second experiment was carried out in which 293 cells were infected with the Ad-sig-ecdhMUC-1/ecdCD40L vector (His tag present), and the proteins fluroescein labeled following purification of the MUC-1/ecdCD40L proteins on a Nickel column. The cells were exposed to a PE conjugated anti-CD11C antibody and to the FITC conjugated ecdhMUC-1/ecdCD40L proteins. The results (see Figure 1D: Panel C) show that the DCs bind the ecdhMUC-1/ecdCD40L proteins.

Fig 1D: Panel C

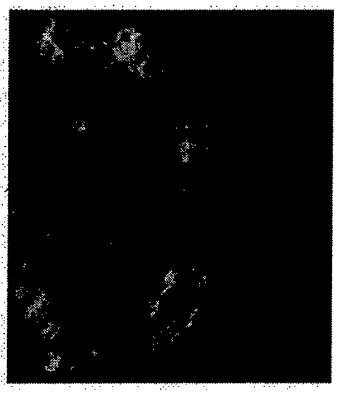


The E7/ecdCD40L Protein Can Be Detected In Vivo for Up to 10 Days In Vivo Following Subcutaneous Injection of the Ad-sig-E7/ecdCD40L Vector. We then sectioned the skin at the site of intradermal injection of the Ad-sig-E7/ecdCD40L vector to determine the time over which the secretable sig-E7/ecdCD40L protein is being released from vector-infected cells. We double stained these sections with a FITC labeled antibody to the CD40L (CD154), which is stained green in Figure 2A, and DAPI, which stains nuclear DNA blue in Figure 2A. As shown in Figure 2A, the double-staining showed that the TAA/CD40L protein binds in vivo to cells near the vector infected cells

for up to 10 days after subcutaneous injection with the Ad-sig-E7/ecdCD40L vector which carries the secretable TAA/ecdCD40L transcription unit. In contrast, a much lower level of double stained positive cells was observed in the epidermis 3 days following injection of the Ad-E7/wtCD40L which contains a non-secretable CD40L transcription unit (data not shown).

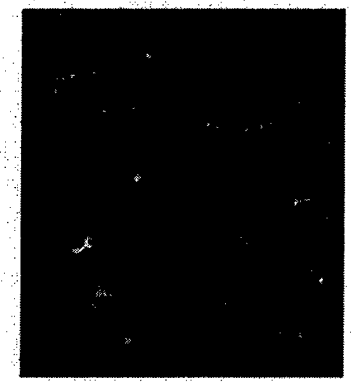
Fig 2A: Panels A and B

PANEL A



Skin section stained by Anti-CD154
5 days after subcutaneous injection
of Ad-sig-E7/ecdCD40L

PANEL B



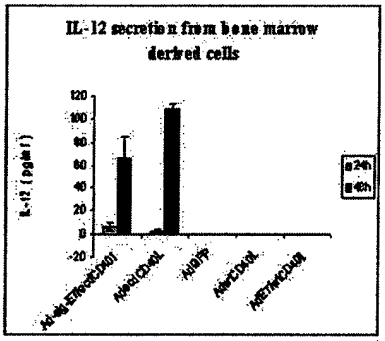
Skin section stained by Anti-CD154
10 days after subcutaneous injection
of Ad-sig-E7/ecdCD40L

Activation of DCs by the Ad-sig-E7/ecdCD40L Vector. As shown in Panel A of Figure 2B, there is a statistically significant increase in the level of induction of IL-12 production following in vitro exposure of the DCs to the Ad-sig-E7/ecdCD40L vector, which carries a transcription unit encoding a secretable TAA/CD40L protein, as compared to vectors encoding a non secretable TAA/CD40L protein, such as the Ad-E7/wtCD40L vector ($P < 0.0001$). 6 ± 3 pg /2×10^5cell / ml/24h and 66 ± 18pg/2×10^5cell /

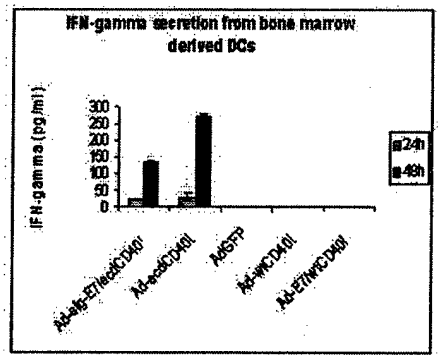
ml/48h of IL-12 were produced by the DCs exposed to the Ad-sig-E7/ecdCD40L vector, whereas exposure of DCs to the Ad-E7/wtCD40L vector, produced no measurable IL-12 at 24 hours or 48 hours.

Figure 2B: Panel A and B

PANEL A



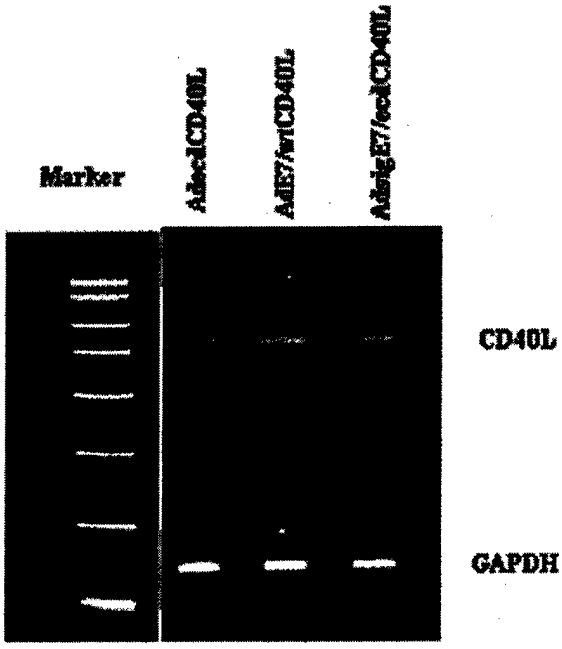
PANEL B



Similarly, there is a statistically significant increase in the IFN-γ released from DCs exposed to the supernatant from the Ad-sig-E7/ecdCD40L vector infected cells: 24$\pm$ 3 pg in the first 24 hours and 132$\pm$ 6 pg during the next 24 hours, as compared to 0 pg released from DCs exposed to supernatant from 293 cells infected with either non secretable CD40L vectors or other control vectors (Panel B of Figure 2B). These experimental data suggest that the TAA/ecdCD40L fusion protein secreted from the Ad-sig-TAA/ecdCD40L infected cells is binding to the CD40 receptor on DCs to generate the observed effect on cytokine release.

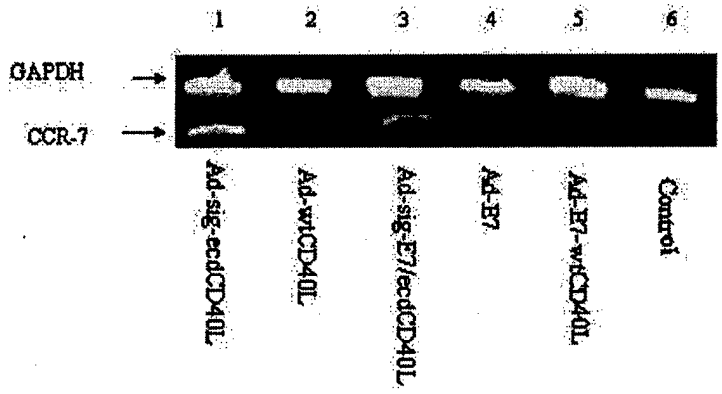
The differences between the cytokine release induced in bone marrow derived dendritic cells exposed to the supernatant from 293 cells infected with CD40L secretable vs non secretable transcription units could be due to differences in the levels of E7/CD40L RNA levels generated by the Ad-sig-E7/ecdCD40L (encoding the secretable E7/CD40L protein) vs the Ad-E7/wtCD40L (encoding the non-secretable E7/CD40L protein). The other possibility is that one vector encodes a secretable vs a non-secretable protein. In order to test this question, RNA was extracted from 293 cells which had been infected by either the Ad-sig-E7/ecdCD40L vector or the Ad-E7/wtCD40L vector at MOI=10. The cDNA was synthesized by using the superscript first-strand system (Invitrogen, Carlsbad, CA). The RT-PCR was performed using 5ug of total RNA extracted from the vector infected cells and the reverse transcription reaction with a random primer. The cDNA product was split into two halves, one of which was used as a template for a PCR reaction with primers specific for the E7/CD40L cDNA and the other half to prime a PCR reaction with primers specific for GAPDH as a control. The results shown below in Figure 2B Panel C, there is no difference in the E7/CD40L mRNA levels using the secretable vs the non-secretable vectors. Thus, it appears that the cytokine release is greater from the bone marrow derived DCs exposed to the supernatant from 293 cells infected with the Ad-sig-E7/ecdCD40L vs the Ad-E7/CD40L vector due to the secretable nature of the E7/CD40L protein from the Ad-sig-E7/ecdCD40L infected cells.

Figure 2B: Panel C



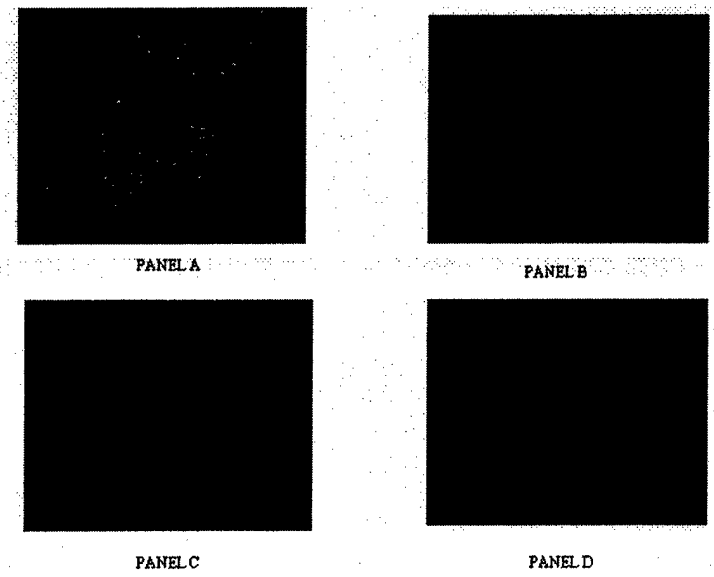
In Vitro and In Vivo Exposure of DC's to the Ad-sig-E7/ecdCD40L Vector Elevates CC Chemokine Receptor-7 (CCR-7) Expression in Mature DCs, and Induces Migration of DCs to Regional Lymph Nodes. Upon antigen exposure, DCs become activated, express CCR-7 and migrate in response to differential gradients of the chemokine ligands CCL 19 and CCL 21 (26). Therefore, we investigated the effect of exposing DCs to supernatants from Ad-sig-E7/ecdCD40L infected 293 cells to determine if the level of CCR-7 expression increased. As shown in Figure 2C, the level of CCR-7 mRNA in DCs increased significantly when DCs were cultured with supernatants from Ad-sig-E7/ecdCD40L or Ad-sig-E7/ecdCD40L vector infected 293 cells (26).

Figure 2C



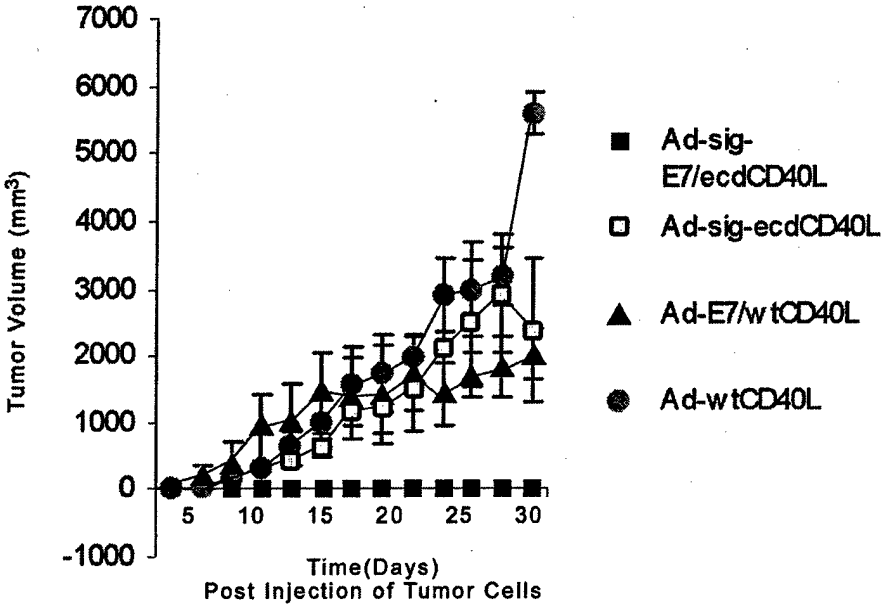
In order to formally test if the subcutaneous injection of the Ad-sig-E7/ecdCD40L vector induces migration of the DCs to the regional lymph nodes in vivo (26), $1X10^6$ DCs were loaded with the CFDA SE dye and exposed to adenoviral vectors at MOI 200. Then, the dye loaded DCs were injected into the left flank of the C57BL/6 mice. Three days after these injections, the mice were sacrificed and the regional axillary lymph nodes on the side of the injection were harvested and studied for the presence of the dye loaded DCs. As shown by the green dots visible in Panel A of Figure 2D, CFDA SE stained DC's are detectably present in the regional lymph nodes following injection of the vector carrying the secretable E7/ecdCD40L transcription unit, whereas no other vector (Panels B-D of Figure 2D) was associated with detectable fluorescent labeled DCs in the regional lymph nodes. No CFDA SE-labeled cells were observed in the non-draining contra-lateral lymph nodes. One of the sections was stained with PE-labeled CD11C antibody to confirm that the green stained cells are DCs (data not shown).

Figure 2D



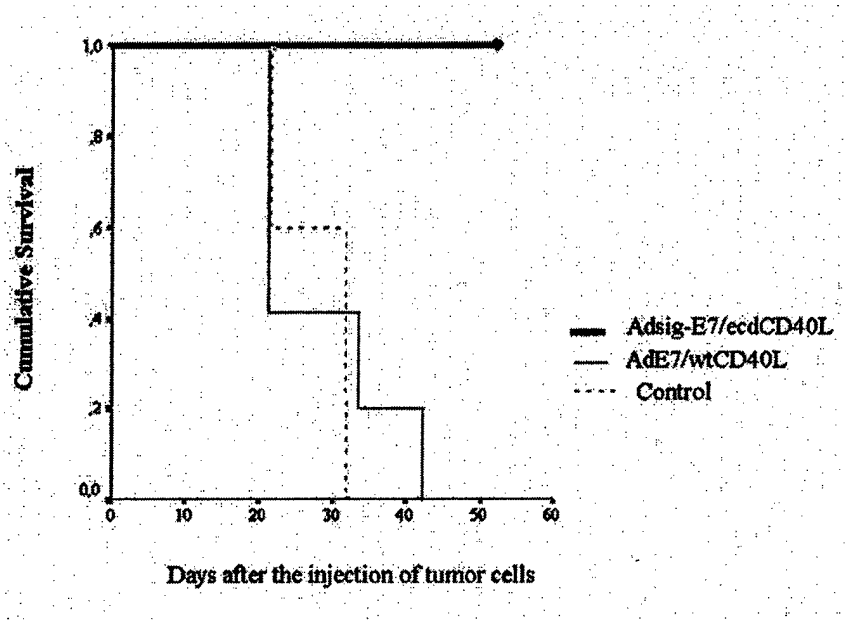
Injection of Ad-sig-E7/ecdCD40L Suppresses Growth of E7 Positive Cancer Cells in Syngeneic Mice. To assess the effect of the subcutaneous injection of the Ad-sig-E7/ecdCD40L vector on the engraftment of the E7 positive TC-1 cell line in C57BL/6 mice, we injected $1 \cdot 10^8$ pfu of each vector subcutaneously into each animal. The mice were vaccinated again one week later with the same vector. Once week after this boost, 5×10^5 E7 positive TC-1 cells were injected subcutaneously on the back of the C57BL/6 mice at a site which was different from that of the vector injections. All of the mice injected with the Ad-sig-E7/ecdCD40L vector remained tumor free throughout the study (up to 18 days after injection), whereas mice injected with all other vectors listed in Figure 3A, including the Ad-E7/wtCD40L vector, which did not carry a secretable TAA/CD40L transcription unit, developed measurable tumors within 13 days after tumor challenge (see Figure 3A).

Figure 3A



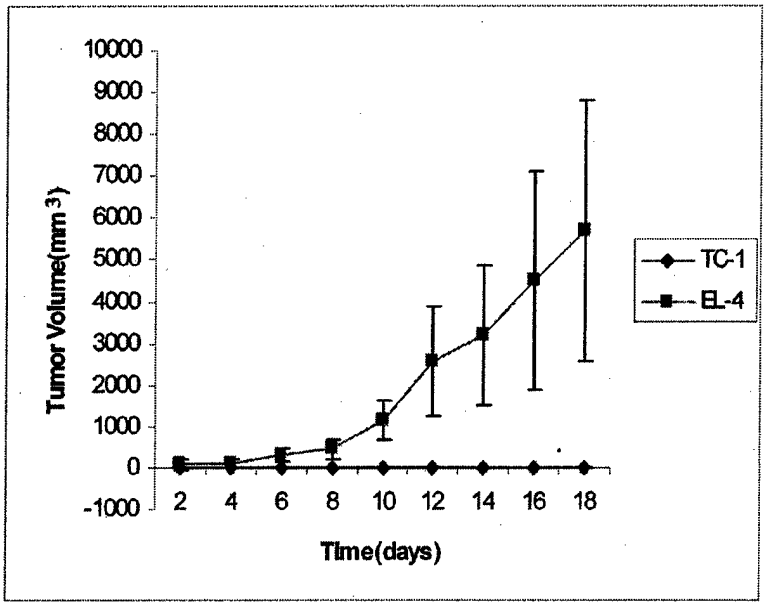
As shown in Figure 3B, the survival of the mice injected with the Ad-sig-E7/ecdCD40L vector (bold unbroken line at the top of the graph) and then injected with the E7 positive TC-1 cells was superior to the survival of mice injected either with the Ad-E7/wtCD40L vector (thin unbroken line), which does not encode a secretable E7/CD40L protein, or injected with no vector (thin broken line), and then injected with the TC-1 cells.

Figure 3B



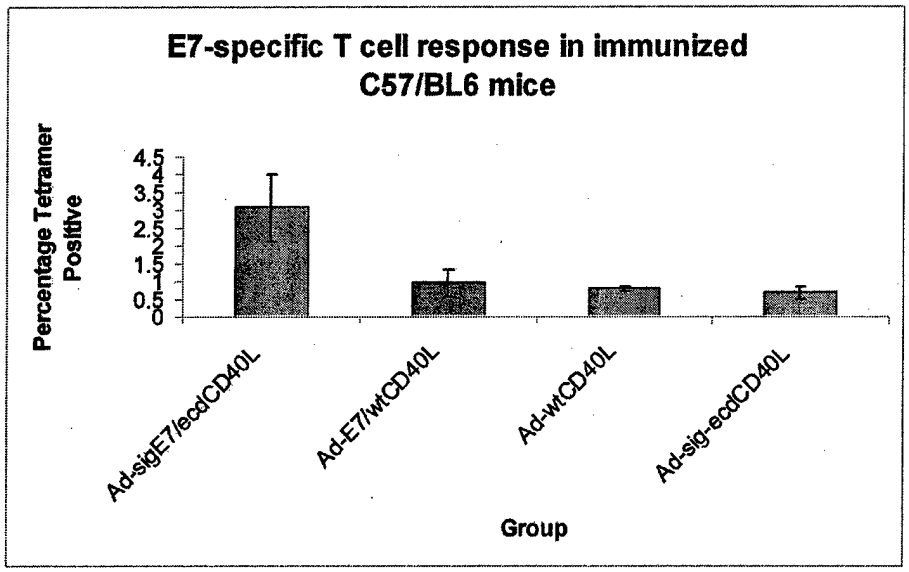
We then tested if the induction of resistance to engraftment of the E7 positive TC-1 cells was specific for the E7 antigen. As is shown in Figure 3C, the subcutaneous injection of the Ad-sig-E7/ecdCD40L vector did not protect mice against the engraftment of E7 negative EL-4 cells but did protect against engraftment of the E7 positive TC-1 cells.

Figure 3C



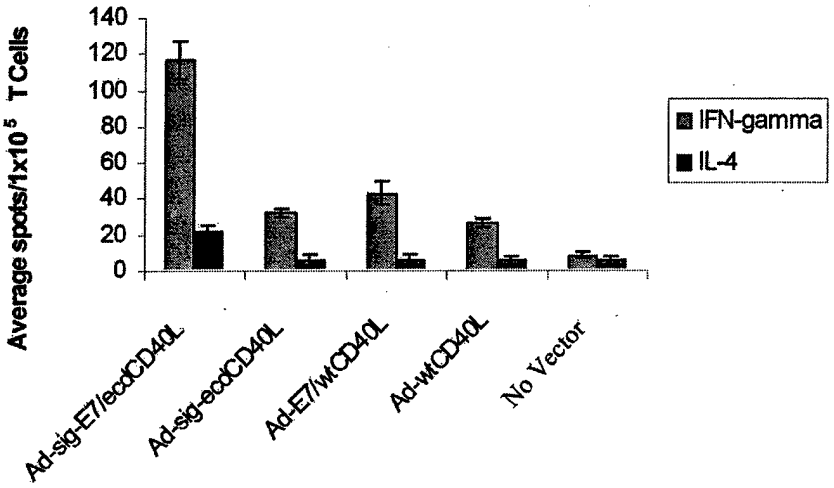
Mechanism of Suppression of E7 Positive Tumor Cells by Ad-sig-E7/ecdCD40L Vector Injections. The spleens were harvested ten days after vector vaccination, and the percentage of $E7_{49-57}$ peptide specific CD8+ T cells was determined by H-$2D^b$ tetramer staining. As shown in Figure 3D, the level of E7 peptide specific T cells in the spleen cells from Ad-sig-E7/ecdCD40L injected animals was increased three fold as compared to the level observed following injection with other vectors including the Ad-E7/wtCD40L vector.

Figure 3D



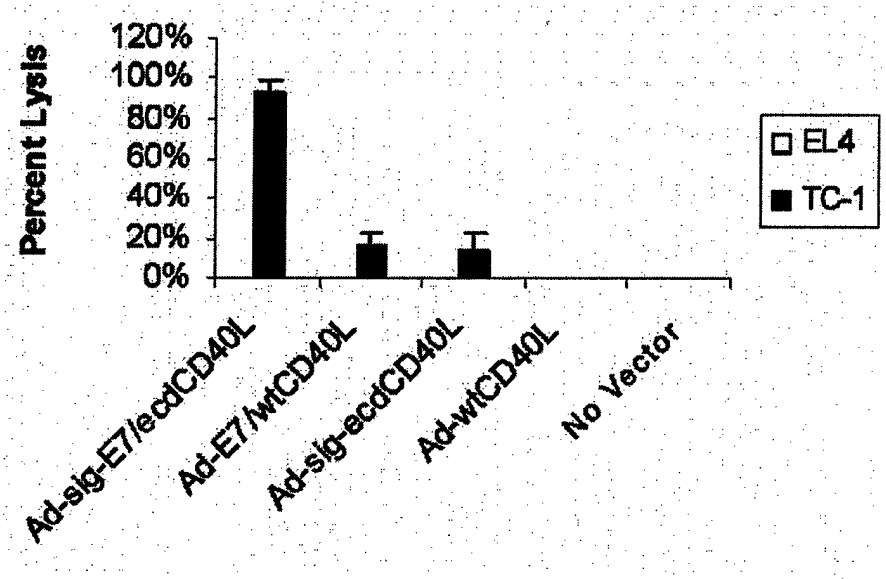
The frequency of IFN gamma and IL-4 secreting T cells from the spleens of mice vaccinated with the various vectors was determined by ELISPOT assays (27). As shown in Figure 3E, mice injected with the Ad-sig-E7/ecdCD40L vector had a greater number of IFN-gamma –secreting T cells (117 $\pm$10.6 spots/1 X 10^5 spleen cells) than mice injected with the vector carrying the non-secretable E7/wtCD40L transcriptional unit (26.3$\pm$ 2.4 spots/1 X 10^5 spleen cells) or any of the other control vectors tested ($p \leq 0.05$). The number of splenic T cells producing a Th2 cytokine (IL-4) was only (22.3$\pm$ 3.68 spots/1 X 10^5 spleen cells). This data indicates that the Ad-sig-E7/ecdCD40L vector vaccination stimulates a Th1 rather than a Th2 immune response.

Figure 3E



Spleen cells from mice injected with the Ad-sig-E7/ecdCD40L vector were pre-stimulated in vitro for 7 days with TC-1 positive cells and then mixed in a 100/1 ratio with E7 positive TC-1 cells in a cytoxicity assay described in the methods section. These studies showed that the splenic T cells from the Ad-sig-E7/ecdCD40L vector sensitized animals lysed 90% of the TC-1 target cells (see Figure 3F). In contrast, spleen cells from uninjected mice or mice injected with the Ad-E7/wtCD40L vector lysed 0% or 20% of the target cells respectively.

Figure 3F



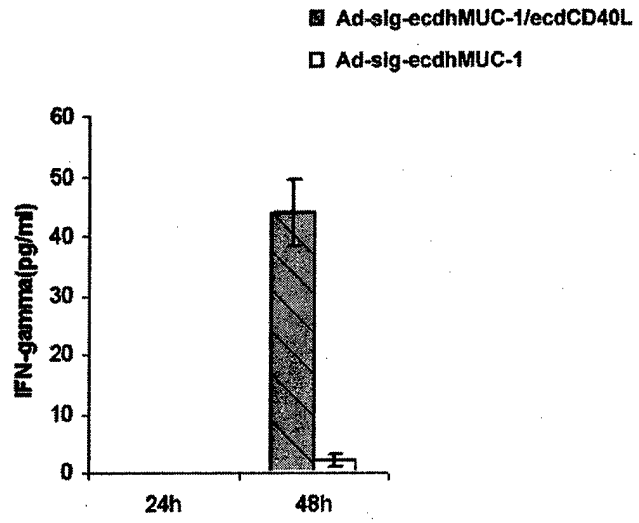
In order to test whether the induced cytolytic immune response was mediated through an HLA restricted process, we added anti-class I antibody or an isotype-matched control antibody to the mixture of effector spleen cells from Ad-sig-E7/ecdCD40L vector injected mice and E7 positive TC-1 target cancer cells. The addition of the anti-HLA antibody suppressed the cytotoxicity to the TC-1 target cells to 10.32%, which is significantly lower than the cytotoxicity found with control antibody (76.91%).

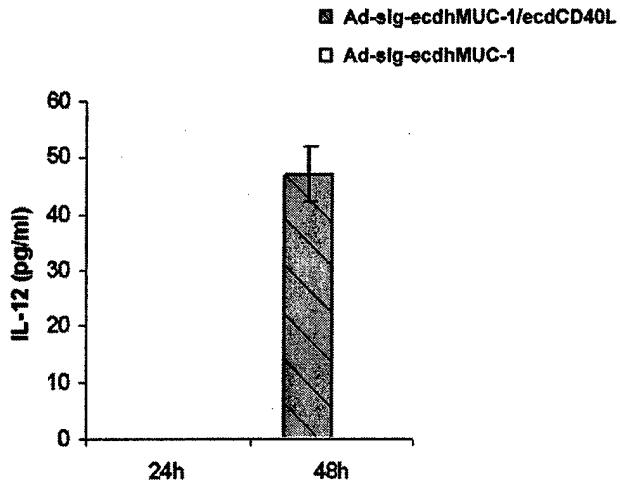
Injection of the Ad-sig-ecdhMUC1/ecd/CD40L Vector Overcomes Anergy to hMUC1 Positive Cells in Mice Which are Transgenic for the Human MUC1 Gene.

We first exposed bone marrow derived DCs to the Ad-sig-ecdhMUC1/ecdCD40L vector, or to the Ad-sig-ecdhMUC1 vector. As shown in Figures 4A and 4B, the ecdhMUC1/ecdCD40L fusion protein can significantly increase the levels of

IFN-gamma and IL-12 cytokines secreted from DCs harvested from the hMUC1.Tg transgenic mice at 48 hours after exposure to the vector. These studies suggest that the ecdhMUC1/ecdCD40L fusion protein can bind to the CD40 receptors on DCs and induce DC activation.

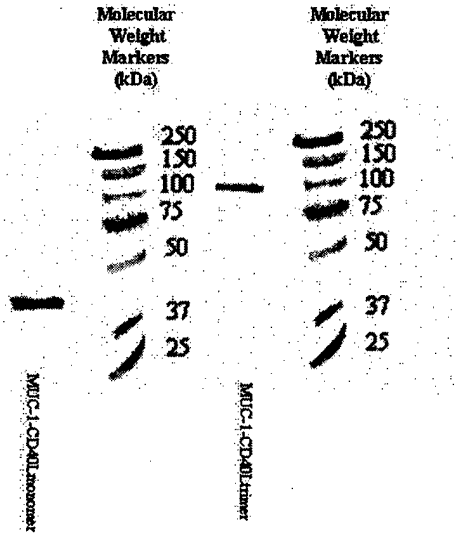
Figures: 4A and 4B





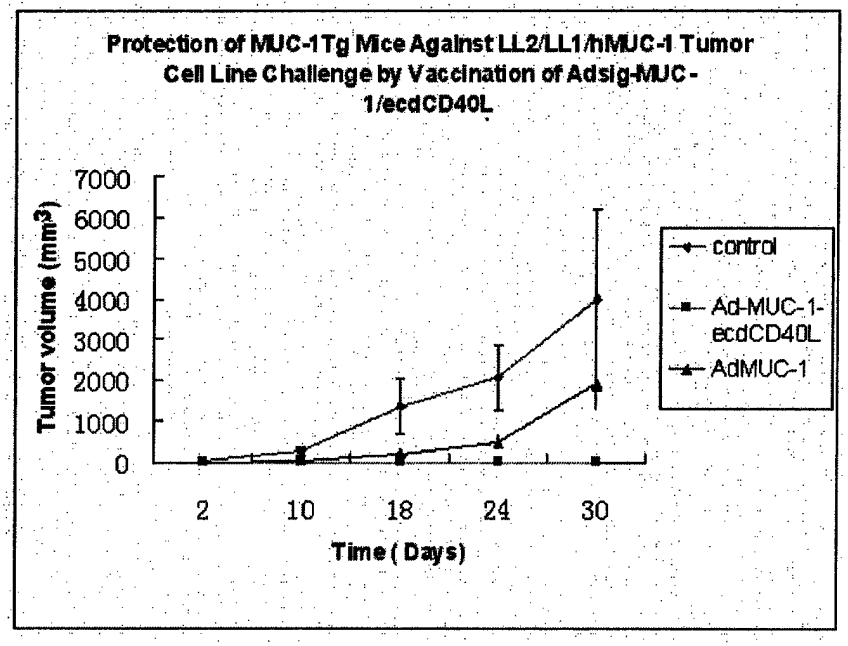
Testing for Functional Trimers of ecdhMUC1/ecdCD40L Proteins Induced by the Ad-sig-ecdhMUC1/ecdCD40L Vector Injections Which Can Activate DCs. In order to formally test if trimeric ecdhMUC1/ecdCD40L proteins are released following infection of cells with the Ad-sig-ecdhMUC1/ecdCD40L vector, we purified (using a His Tag purification kit) the ecdhMUC1/ecdCD40L protein from the supernatant of 293 cells exposed to the Ad-sig-ecdhMUC1/ecdCD40L. In this vector, an HSF1 trimer stabilization domain had been placed between the ecdhMUC1 and ecdCD40L fragments and a His tag was placed at the carboxyl terminal domain of the ecdCD40L protein. As shown in Figure 4C, the molecular weight of the ecdhMUC1/ecdCD40L protein under non-denaturing conditions was close to 3 times that seen under denaturing conditions. This experiment showed that trimers could be formed by the ecdhMUC1/ecdCD40L fusion protein.

Figure 4C



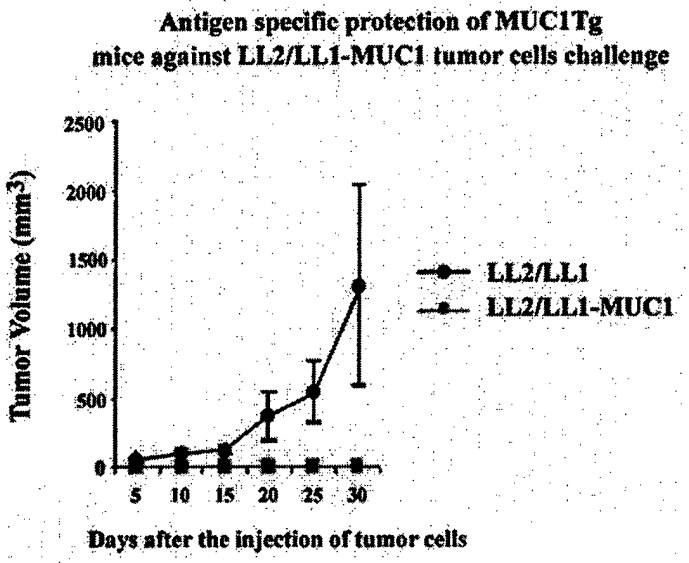
Subcutaneous Injection of the Ad-sig-ecdhMUC1/ecdCD40L Vector Overcomes Anergy for hMUC1 Positive Cells in Mice, which are Transgenic for hMUC1. As shown in Figure 5A, mice injected subcutaneously with the Ad-sig-ecdhMUC1/ecdCD40L vector (solid squares) were resistant to engraftment by the hMUC1 positive LL2/LL1hMUC1 mouse cancer cells whereas mice vaccinated with the Ad-sig-ecdhMUC-1 vector (solid triangles) or the untreated control animals not injected with a vector (solid diamonds) were not resistant to the growth of the same cells. This data shows that the full chimeric hMUC-1/ecdCD40L transcription unit is needed for complete suppression of the growth of the hMUC-1 cell line in the hMUC-1.Tg mice.

Figure 5A



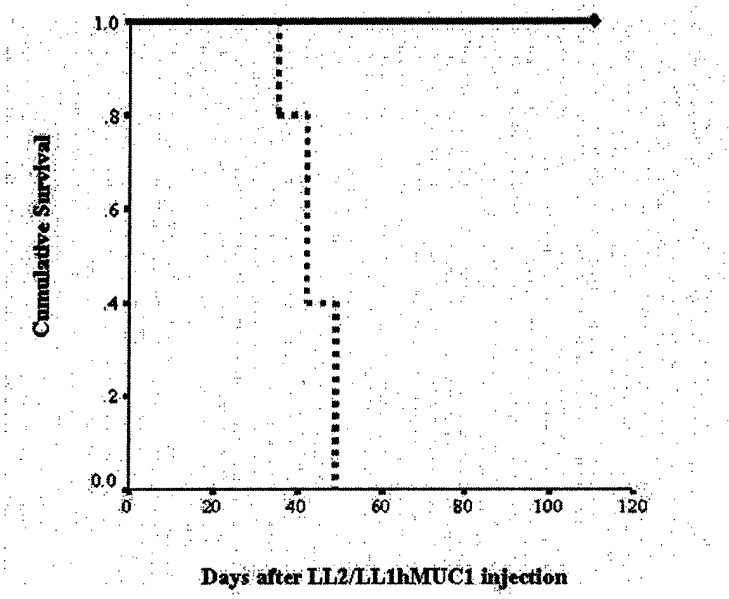
The mice injected with the Ad-sig-ecdhMUC1/ecdCD40L vector suppressed the growth of the hMUC1 antigen positive LL2/LL1hMUC1 cell line whereas this same vector did not suppress the growth of the parental cell line (LL2/LL1), which was not positive for the hMUC1 antigen (see Figure 5B).This showed that the immune response was antigen specific.

Figure 5B



As shown in Figure 5C, the mice injected with the Ad-sig-ecdhMUC1/ecdCD40L vector (see solid bold line at the top in Figure 5C) lived longer than did the mice injected with a control vector (see broken line in Figure 5C) and then injected subcutaneously with the LL2/LL1hMUC1 cell line.

Figure 5C

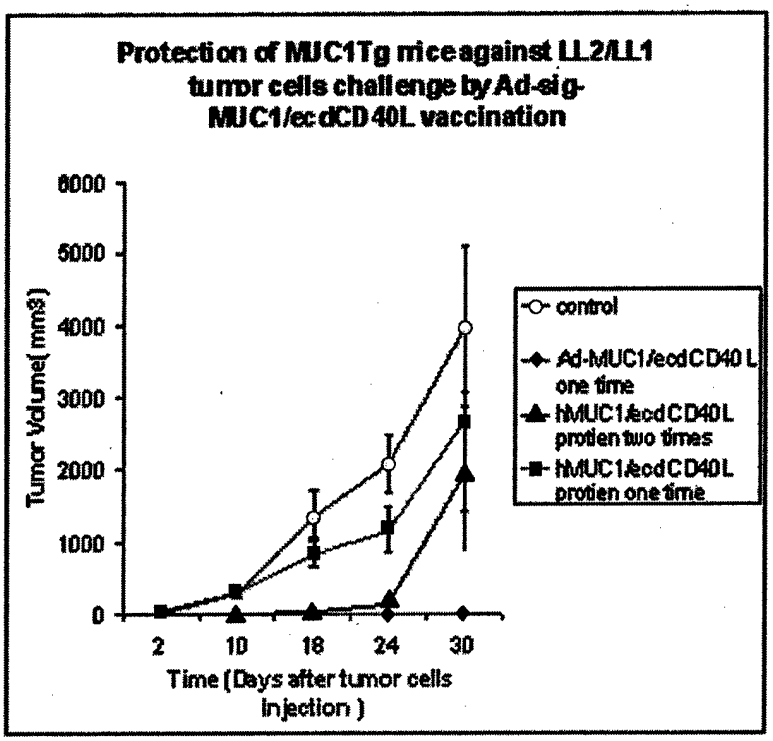


Study of the Cellular Mechanisms Through Which Ad-sig-ecdhMUC1/ecdCD40L Subcutaneous Injections Overcome Anergy.

A. Will the Injection of the ecdhMUC1/ecdCD40L Protein Overcome Anergy in the hMUC1.Tg Mouse Without the Vector "Danger Signal"? One question is whether the subcutaneous injection of the ecdhMUC1/ecdCD40L protein would induce the cellular immune response that was seen with the Ad-sig-ecdhMUC1/ecdCD40L vector injections. As shown by the data in Figure 6A, subcutaneous injection of the ecdhMUC1/ecdCD40L protein did not induce an immune response that could protect the hMUC1.Tg mice from the growth of the LL2/LL1hMUC1 cell line. It is possible that the use of the adenoviral

vector injections provide the "danger signal" (2) necessary to induce the immune response in the hMUC1.Tg mice.

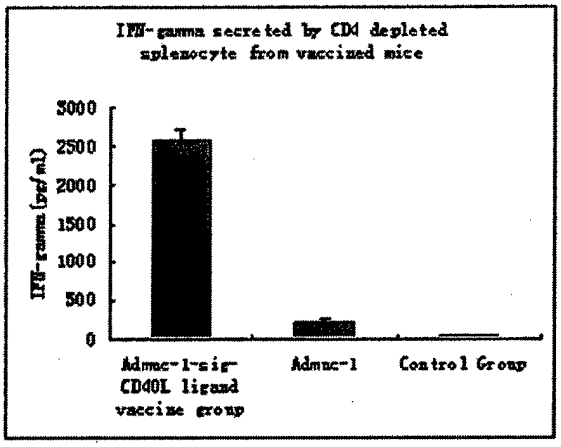
Figure 6A



B. Cytokine Release from Vaccinated vs Non Vaccinated Mice. In order to test if the Ad-sig-ecdhMUC1/ecdCD40L induction of cellular immunity was mediated by CD8 T cells, the spleen T cells of the Ad-sig-ecdhMUC1/ecdCD40L vector vaccinated hMUC-1.Tg mice or the Ad-sig-ecdhMUC-1 vaccinated mice were depleted of CD4 T cell lymphocytes with magnetic beads. As shown in Figure 6B, the CD8 T cell lymphocytes

isolated 7 days following injection from the spleens of hMUC1.Tg mice with the Ad-sig-ecdhMUC1/ecdCD40L vector released over 2500 times the level of IFG-gamma as did CD8 T cells which were taken from control vector vaccinated MUC1.Tg mice and 50 times the levels of IFG-gamma as did mice vaccinated with the Ad-sig-ecdhMUC-1 vector.

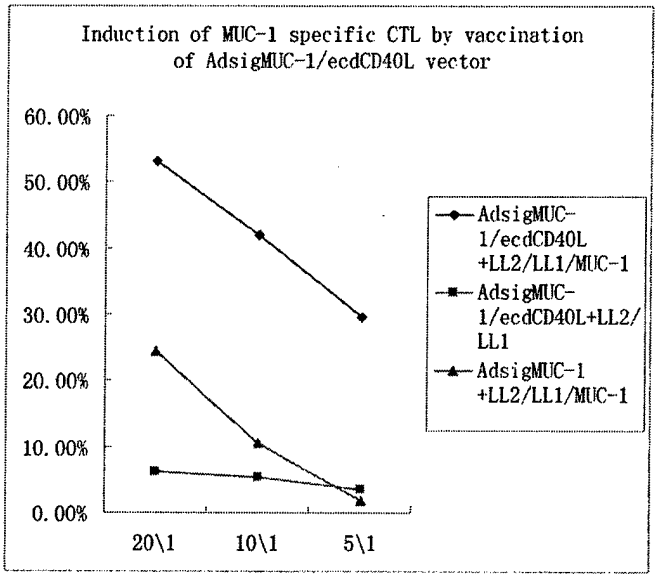
Figure 6B



C. Cytotoxicity Assay of Splenic T Cells From Ad-sig-ecdhMUC1/ecdCD40L Vector Injected Mice Against LL2/LL1hMUC1 or LL2/LL1 Cancer Cells. Splenic T cells were collected from hMUC1.Tg mice 7 days following injection with the Ad-sig-ecdhMUC1/ecdCD40L vector or the Ad-sig-ecdhMUC-1 vector and were then exposed to the hMUC1 antigen positive LL2/LL1hMUC1 cancer cells for 7 days. Then the stimulated T cells were mixed in varying ratios with either the hMUC1 positive LL2/LL1hMUC1 cells or the hMUC1 negative LL2/LL1 cancer cells. As shown in

Figure 6C, the T cell from Ad-sig-ecdhMUC1/ecdCD40L vaccinated mice can specifically kill the cancer cells carrying the hMUC1 antigen but not the antigen negative cells. Moreover, the level of hMUC-1 specific cytotoxic T cells in the Ad-sig-ecdhMUC-1/ecdCD40L mice were 6 times higher than in mice vaccinated with the Ad-sig-ecdhMUC-1 vector.

Figure 6C

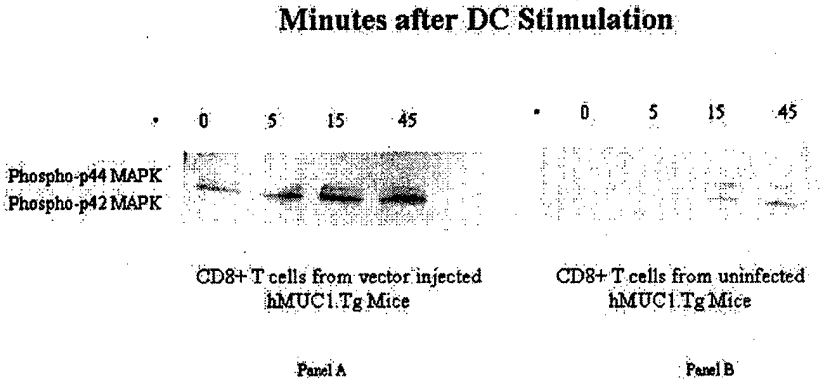


D. Ad-sig-ecdhMUC1/ecdCD40L Vector Injection Overcomes Resistance to Expansion of hMUC1 Specific T Cells. Although anergic peripheral CD8+ T cells can be induced to lyse target cells in an antigen specific manner, they have been found to exhibit a block in the activation of the ERK proliferation signal transduction pathway following antigenic stimulation (28). To determine if the CD8 cells from hMUC1.Tg

mice expressed the active form of ERK1/2 upon vector immunization, splenic CD8 positive T cells were obtained from non injected hMUC1.Tg transgenic mice or mice injected 7 days previously with the Ad-sig-ecdhMCU1/ecdCD40L vector, and stimulated in vitro with the Ad-sig-ecdhMUC1/ecdCD40L vector infected DCs.

CD8 T cells from unvaccinated hMUC1.Tg mice showed delayed kinetics and decreased total phosphorylation of ERK1 and ERK2 proteins (see Panel B of Figure 6D), as compared to CD8 T cells from Ad-sig-ecdhMUC1/ecdCD40L vaccinated hMUC1.Tg mice (see Panel A of Figure 6D). This data suggests that Ad-sig-hMUC1/ecdCD40L vector injection induces an antigen-specific CD8 T cell immune response to the MUC1 self-antigen through activation of the proliferation induction pathways in CD8 T cells.

Figure 6D



Discussion

Our goal was to characterize the steps through which the vaccination of mice with the Ad-sig-TAA/ecdCD40L vector can induce an immune response to TAA positive cells in anergic animals. Our experimental results suggest that the subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector leads to the continuous release of the TAA/ecdCD40L protein for at least a 10-day period. The binding of this protein to DCs induces increased levels of secondary signals of activation (CD80 and CD86) as well as the CCR-7 chemokine receptor on DCs which leads to the migration of the TAA loaded DCs to the regional lymph nodes. These events induce up to an increase in the levels of the TAA specific CD8+ cytotoxic T lymphocytes in the spleens of Ad-sig-TAA/ecdCD40L vector injected mice.

This increase in the TAA specific CD8+ lymphocytes in the Ad-sig-ecdhMUC1/ecdCD40L vector injected mice overcomes the anergy that exists to the hMUC1 antigen in hMUC1.Tg mice, which have expressed the hMUC1 antigen since birth. These experiments further show that the induction of immunity is associated with the release of Th-1 cytokines, is HLA restricted, and is accompanied by an increase in the total phosphorylation of ERK1 and ERK2 pathways in T cells from vector injected hMUC1.Tg mice when the T cells are exposed to Ad-sig-ecdhMUC1/ecdCD40L vector infected DCs.

In contrast to the subcutaneous injection of the Ad-sig-ecdhMUC1/ecdCD40L vector, the subcutaneous injection of the ecdhMUC1/ecdCD40L protein does not induce immune

protection against the growth of the hMUC1 positive LL1/L2hMUC1 tumor cells (see Figure 6A). This suggests that the "danger signal" (2) associated with the adenoviral vector carrying the ecdhMUC1/ecdCD40L transcription unit, is an important part of overcoming the anergy to the hMUC1 antigen which exists in the hMUC1.Tg mice.

The oral TAA/CD40L Salmonella typhimirium DNA vaccine of Xiang and co-workers (14) had three potential limitations: 1. the need to use targeted IL-2 in addition to the oral DNA bacterial vaccine; 2. the use of a DNA vaccine which due to its inefficiency of transfection, generates only low levels of expression for a short period of time, and 3. the need to restrict the vaccination to the development of the antigen loaded and activated DCs to the secondary lymphoid tissue of the gastrointestinal tract. The restriction to the T cells of the secondary lymphoid tissue of the gastrointestinal tract (29) in the method of Xiang et al (14) could be a limitation.

Since the adenoviral vector used in our work (see reference 1 and the results reported in this paper) can be administered to any part of the body, the homing of the T cells to the region of origin could be directed to the secondary lymphoid organs of any tissue by selection of the site of injection. In contrast to the method of Xiang et al (14), there was no need to follow the vaccination of the mice with targeted IL2 treatment to break tolerance or to induce resistance to the engraftment of cancer cell lines in 100% of the vaccinated mice in our studies, as was the case in the studies of Xiang et al (14). Finally, we showed that the subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector is able

to overcome the anergy that develops to tumor associated antigens, which are present from birth.

There are many reasons for having selected an in vivo method of activating and TAA loading DCs. The first is that the goal of the paper was to study the steps involved in the in vivo activation and antigen loading of DCs, not to compare the in vivo vs the ex vivo loading of DCs. The in vivo activation was an attractive option to study for the following reasons: 1. The work of Xiang et al (14) with the TAA/CD40L DNA vaccine involved in vivo vaccination, not ex vivo loading and activation. We wanted to determine if we could improve upon the in vivo activation and TAA loading seen when an adenoviral vaccine was used instead of a DNA vaccine. 2. The in vivo activation by one or two subcutaneous injections of a vector could turn out to be vastly cheaper and simpler to administer than complex strategies involving ex vivo activation and TAA loading of DCs. 3. The in vitro activation approach is limited by the numbers of DCs that can be produced, the fact that the culture systems in vitro can never duplicate the in vivo environment, and the in vivo approach was shown to continue the TAA/CD40L protein release over a 10-14 day period whereas the ex vivo approach involves just a single injection. Finally, clinical trials involving ex vivo activation or tumor antigen loading of DCs have by and large been proven to be less effective than in vivo methods of vaccination (13).

A notable finding was that control experiments with vectors encoding just the TAA alone or the CD40L alone were not as effective in activating DCs or inducing a cellular immune response against TAA positive cancer cells in animal models. The question may

be asked why the vaccination with vectors encoding the secretable fusion protein of the TAA/CD40L is more effective in inducing an immune response than vectors containing either the TAA alone or the CD40L alone. We have shown in this paper that the chimeric TAA/CD40L fusion protein can form functional trimers, a requirement for binding of the CD40L end of the fusion protein to the CD40 receptor on the DCs. Once the chimeric protein binds to the DCs, two things happen: 1. the DCs are activated so as to be effective in providing the CD8 cells with the secondary signals necessary to activate CD8 TAA specific T cells; 2. The chimeric TAA/CD40L protein is taken up into the DCs by endocytosis thereby permitting the TAA to be processed in a way which results in its being available for presentation by Class I MHC molecules. The fact that individual DCs are both activated and TAA loaded is the advantage of the vectors encoding the TAA/CD40L fusion protein.

The immune response induced by the subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector is antigen specific, is dependent on the activation of the DCs in and around the vector injection site, and on the migration of the TAA loaded and activated DCs to the regional lymph nodes. It is not possible to overcome anergy with subcutaneous injection of the TAA/ecdCD40L protein or the subcutaneous injection of an adenoviral vector that carries a transcription unit encoding a non-secretable TAA/ecdCD40L protein. These experimental results suggest that this approach to the activation of the immune response against tumor cells merits further study in pre-clinical and clinical models.

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References

1. Zhang L, Tang YC, Akbulut H, Zelterman D, Linton PJ, and Deisseroth AB. An adenoviral vector cancer vaccine that delivers a tumor-associated antigen/CD40-ligand fusion protein to dendritic cells. Proc. Natl. Acad. Sci. USA (2003); 100:15101-15106.

2. Matzinger P. Tolerance, Danger and the Extended Family. Annual. Rev. Immunol. (1994); 12:991-993.

3. Wurtzen PA, Nissen MH, and Claesson MH. Maturation of dendritic cells by recombinant human CD40L-trimer leads to a homogeneous cell population with enhanced surface marker expression and increased cytokine production. Scand J Immunol. (2001); 53: 579-87.

4. Grewal I and Flavell R. CD40 and CD154 in cell-mediated immunity. Annual Rev. Immunol. (1998); 16:111-135.

5. Bennett S, Carbone F, Karamalis F, Flavell R, Miller J, and Heath W. Help for cytotoxic T-cell responses are mediated by CD40 signaling. Nature (1998); 393: 478-480.

6. Schoenberger S, Toes RE, van der Voort EI, Offringa R, and Melief C. T-cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions. Nature (1998); 393:480-483.

7. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B and Palucka K. (2000). Immunobiology of Dendritic Cells. Annual Rev. Immunol. 2000; 18: 767-811.

8. Bennett SR, Carbone FR, Karamalis F, Miller JF, and Heath WR. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. J. Exp. Med. 1997;18665-18670.

9. Schuurhuis DH, Laban S, Toes RE, Ricciardi-Castagnoli P, Kleijmeer MJ, van der Voort EI, Rea D, Offringa R, Geuze HJ, Melief CJ, and Ossendorp F. Immature dendritic cells acquire CD8(+) cytotoxic T lymphocyte priming capacity upon activation by T helper cell-independent or –dependent stimuli. J. Exp. Med. 2000;192: 145-150.

10. Fanslow WC, Srinivasan S, Paxton R, Gibson MG, Spriggs MK, and Armitage RJ. Structural characteristics of CD40 ligand that determine biological function. Semin.

Immunol.1994:6:267-278.

11. Tuting T, DeLeo AB, Lotze MT, and Storkus WJ. Genetically modified bone marrow-derived dendritic cells expressing tumor-associated viral or "self" antigens induce antitumor immunity in vivo. Eur. J. Immunol. 1997; 27: 2702-2307.

12. Fong L, Brockstedt D, Benike C Wu L, and Engleman EG. Dendritic cells injected via different routes induce immunity in cancer patients. J. Immunol. 2001;166: 4254-4259.

13. Wherry EJ, Teichgraber V, Becker TC, Masopust D, Kaech SM, Anitia R, von Adnrian UG, and Ahmed R. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nature Immunology (2003); 4:225-234.

14. Xiang R, Primus FJ, Ruehlmnn JM, Niethammer AG, Silletti S, Lode HN, Dolman CS, Gillies SD, and Reisfeld RA. Dual function DNA vaccine encoding carcinoembryonic antigen and CD40L trimer induces T cell-mediated protective immunity against colon cancer in carcinoembryonic antigen-transgenic mice. Journal Immunology 2001;167:4560-4565.

15. Karpuses M, Hsu YM, Wang JH, Thompson J, Lederman S, Chess L, and Thomas D. All the sequences necessary to stabilize the CD40L trimer are contained in the extracellular domain. Structure 1995; 3:1031-1039.

16. Kaplan JM, Yu Q, Piraino ST, Pennington SE, Shankara S, Woodworth LA, and Roberts BL. Induction of antitumor immunity with dendritic cells transduced with adenovirus vector-encoding endogenous tumor-associated antigens. J. Immunol. 1999; 163: 699-707.

17. Berry JM, and Palefsky JM. A review of human papillomavirus vaccines: from basic science to clinical trials. Frontiers of Bioscience 2003; 8: 333-345.

18. Liu W, Gao F, Zhao K, Zhao W, Fernando G, Thomas R, and Codon RI. Modified human papillomavirus Type 16 E7 DNA vaccine enhances cytotoxic T-lymphocyte induction and anti-tumor activity. Virology 2002; 15:301-343.

19. Kim TY, Myoung HJ, Kim JH, Moon IS, Kim TG, Ahn WS, and Sin JI. Both E7 and CpG-oligodeoxynucleotides are required for protective immunity against challenge with

human papillomavirus 16 (E6/E7) immortalized tumor cells: involvement of CD4+ and CD8+ T cells in protection. Cancer Research 2003; 63:2393-2398.

20. Hu G, Liu W, Mendelsohn J, Ellis L, Radinsky R, and Deisseroth AB. (1997). High epidermal growth factor-receptor levels induced by the human papilloma viruss E6/E7 proteins lead to unregulated growth in cervical carcinoma cells. Journal of the National Cancer Institute. 1997; 89:1271-1277.

21. Greenlee RT, Murray T, Bolden S, and Wingo, PA. (2000). Cancer Statistics *CA* Cancer J.2000; 50: 7–33.

22. Ren J, Li Y, and Kufe D. Protein kinase C delta regulates function of the DF3/MUC1 carcinoma antigen in beta-catenin signaling. J. Biol. Chem. 2002; 277: 17616-17622.

23. Kontani K, Taguchi O, Narita T, Izawa M, Hiraiwa N, Zenita K, Takeuchi T, Murai H, Miura S, and Kannagi R. Modulation of MUC1 mucin as an escape mechanism of breast cancer cells from autologous cytotoxic T-lymphocytes. Br. J. Cancer 2001; 84:1258-1264.

24 Rowse G J, Tempero R M, VanLith M L, Hollingsworth M A, and Gendler S J. Tolerance and immunity to MUC1 in a human MUC1 transgenic murine model. Cancer Res. 1998; 58: 315-321.

25. He TZ, Zhou S, da Costa LT, Yu J, Kinzler KW, and Vogelstein BA. Simplified system for generating recombinant adenovirus. Proc. Natl. Acad. Sci. USA, 1998; 95:2509-2514.

26. Parlato S, Santini SM, Lapenta C, Di Pucchio T, Logozzi M, Spada, M., Giammarioli, AM, Malorni W, Fais S, and Belardelli F. Expression of CCR-7, MIP-3 beta, and Th-1 chemokines in type I IFN-induced monocyte-derived dendritic cells: importance for the rapid acquisition of potent migratory and functional activities. Blood 2001; 98:3022-3029.

27. Rininsland FH, Helms T, Asaad RJ, Boehm BO, and Tary-Lehmann M. Granzyme B ELISPOT assay for ex vivo measurements of T cell immunity. J. Immunol. Methods 2000; 240:143-155.

28. Ohlen C, Kalos M, Cheng LE, Shur AC, Hong DJ, Carson BD, Kokot NC,

Lerner CG, Sather BD, Huseby ES, and Greenberg PD. $CD8^+$ T Cell Tolerance to a tumor-associated antigen is maintained at the level of expansion rather than effector function. Journal of Experimental Medicine 2002; 195:1407-1418.

29. Gallichan WS, and Rosenthal KLT. Cells home to tissue of body in which the lymph nodes are located in which they were originally activated. J. Exp. Med. 1996; 184: 1879-1890.

30. Zhang L, Akbulut H, Tang YC, Peng XY, Pizzorno G, Manegold S, and Deisseroth A. Adenoviral vectors with E1A regulated by tumor specific promoters are cytolytic for breast cancer and melanoma. Molecular Therapy 2002: 6386-6393.

Figure Legends

Figure 1: TAA/ecdCD40L Protein Produced by Ad-sig-TAA/ecdCD40L Infected Cells Binds to DCs. Fig. 1A. Proposed Mechanism for Induction of Immune Response by the Ad-sig-TAA/CD40L Vector. The injection of Ad-sig-TAA/ecdCD40L induces in vivo activation and tumor antigen loading of DCs, migration of the DCs to regional lymph nodes, and activation of CD8+ cytotoxic T cells, which are specific for cells carrying the tumor antigen. Fig. 1B: In Vitro Expression of the E7/ecdCD40L Transcription Unit. Plasmid expression vectors encoding the non-secretable E7/wtCD40 ligands (Lane 1), the secretable ecd of the CD40 ligand (sig-ecdCD40L) alone (Lane 2), and the secretable sig-E7/ecdCD40 ligand protein (Lane 3) produced in a cell free transcription/translation system are as predicted: Lane 1: E7/wtCD40L is 39 kDa; Lane 2: sig-ecdCD40L is 22 kDa; and Lane 3: sig-E7/ecdCD40L is 32 kDa. The molecular weight markers are at the extreme right hand lane. Fig. 1C: Western blot analysis of the expression of E7/ecdCD40L protein in 293 cells. The molecular weights of the TAA/ecdCD40L proteins produced from 293 cells infected by the Ad-sig-TAA/ecdCD40L vectors adenoviral vectors were as predicted for: Lane 1: lysates from cells infected with the Ad-sig-GFP/ecdCD40L vector; Lane 2: lysates from cells infected with the Ad-sig-E7/ecdCD40L vector; Lane 3: lysates from cells infected with the Ad-sig-ecdCD40L vector; Lane 4: lysates from the Ad-sig-ecdhMUC1/ecdCD40L vector. The molecular weight markers are in the lane at the extreme right hand lane. Fig. 1D: Secretory Form of TAA/ecdCD40L Binds In Vitro to DCs. Bone marrow derived DCs

were fractionated to 78% purity. Panels A and B: FITC labeled E7/ecdCD40L recombinant proteins released from Ad-sig-E7/ecdCD40L infected 293 cells were incubated with bone marrow derived DCs. The cells were photographed with light microscopy (left hand photographs of each panel) to demonstrate the morphology of the DCs, and then with fluorescent microscopy (right hand photograph of each panel) to detect the binding of the fluoresceinated proteins. Panel A: DCs incubated with FITC labeled proteins from the supernatant of cells infected with the Ad-sig-E7/ecdCD40L; Panel B: DCs incubated with FITC labeled proteins from the supernatant of cells infected with the Ad-sig-ecdCD40L vector. Panel C: Proteins released from Ad-sig-ecdhMUC-1/ecdCD40L infected 293 cells were fractionated on a Nickel column to purify the His tagged ecdhMUC-1/ecdCD40L proteins. These proteins were fluorescein labeled as outlined in the methods. Both the FITC labeled ecdhMUC-1/ecdCD40L proteins and a PE-conjugated rat anti-mouse CD11C antibody were added to the purified DCs. The left hand photograph (A)_shows a photograph of the cells exposed to a laser which is excitatory for phycoerythrin. The middle photograph (B) shows the cells exposed to a laser which is excitatory for FITC. The right hand photograph (C) contains an overlay of the images from A and B.

Figure 2: TAA/ecdCD40L Protein from Ad-sig-TAA/ecdCD40L Vector Infected Cells Binds to and Activates DCs Which Induce Migration to Regional Lymphoid Tissue. Fig. 2A. Injection of the Ad-sig-E7/ecdCD40L Vector Generates the Release of the E7/ecdCD40L Protein Around the Vector Injection Site for up to 10 Days. Skin section stained by anti-CD154 and DAPI **5 days** (Panel A) and **10 days** (Panel B) after

injection of the Ad-sig-E7/ecdCD40L vector. Fig. 2B, Panels A and B: Bone Marrow Derived DCs Release IL-12 and IFN-Gamma after Exposure to the Ad-sig-E7/CD40L Vector. IL-12 (Panel A) or IFN gamma (Panel B) released by vector infected DCs into the supernatant medium was measured by ELISA in DCs stimulated for 24 hours (light color) and 48 hours (dark color) with the following adenoviral vectors: Ad-sig-E7/ecdCD40L, Ad-ecdCD40L, Ad-GFP, Ad-wtCD40L, and AD-E7/wtCD40L. Fig. 2B, Panel C: Semi-quantitative RTPCR reaction to measure the levels of E7/CD40L RNA in 293 cells exposed to the Ad-sig-eE7/ecdCD40L vector or the Ad-E7/wtCD40L vector. 293 cells were infected with the following vectors (Ad-sig-ecdCD40L, Ad-E7/wtCD40L, and Ad-sig-E7/ecdCD40L) at MO1 10. Then the RNA was isolated and the PCR reaction was carried out with primers specific for the E7/CD40L mRNA. The cDNA generated was then fractionated on a molecular weight gel. The electrophoretic species corresponding to the predicted molecular weight of the PCR product from the E7/CD40L template is indicated at the right hand margin of the gel by the CD40L label. The electrophoretic mobility of a PCR cDNA product using the same RNA but primers specific for GAPDH (loading control) is indicated at the right hand margin by GAPDH. Fig. 2C. Up-regulation of CCR-7 mRNA in DCs Exposed to the Ad-sig- E7/ecdCD40L Vector. Lane 1: the Ad-sig-ecdCD40L vector; Lane 2: the Ad-wtCD40L vector; Lane 3: the Ad-sig-E7/ecdCD40L vector; Lane 4: the Ad-E7 vector; Lane 5: the Ad-E7-wtCD40L vector; Lane 6: uninfected cells (control). Fig. 2D. In Vivo Study of Migration of DC's to Regional Lymph Nodes Following Loading of DCs with CFDA SE Dye and Infection with the Ad-sig-E7/ecdCD40L Vector. Bone marrow derived DCs were loaded in vitro with the CFDA SE supravital dye, exposed in vitro to the following vectors at

200 MOI: Panel A: Ad-sig-E7/ecdCD40L; Panel B: Ad-ecdCD40L; Panel C: Ad-E7/wtCD40L; Panel D: Ad-wtCD40L. The DCs were then injected subcutaneously into the hind flank of the test mice. Two days later, the regional lymph nodes were dissected and frozen sections were studied with a fluorescent microscope.

Figure 3: Mechanism of the Ad-sig-E7/ecdCD40L Vector Induced Suppression of the Growth of E7 Positive TC-1 Tumor Cells in C57BL/6 Mice. Fig. 3A. Resistance to the Subcutaneous Growth of 5 X 10^5 E7 Positive TC-1 Cancer Cells in Mice Following Two Injections with 1 X 10^8 pfu of the Ad-sig-E7/ecdCD40L Vector 7 Days Apart. Solid squares: Ad-sig-E7/ecdCD40L; Open squares: Ad-sig-ecdCD40L; Solid triangles: Ad-E7/wtCD40L; and Solid circles: Ad-wtCD40L. Fig. 3B. Survival of Mice Vaccinated with Ad-sig-E7/ecdCD40L Vectors. The following vectors were injected into C57BL/6 mice following which the E7 positive TC-1 cancer cells were injected into the subcutaneous space of the mice. Bold continuous line: mice treated with two subcutaneous injections seven days apart of 1 X 10^8 pfu of the Ad-sig-E7/ecdCD40L vector; Thin continuous line: mice treated with subcutaneous injections of the Ad-wtCD40L vector; Broken thin line; control mice, which were not treated with vector injections. Fig. 3C. Comparison of the Effect of Two Subcutaneous Injections of 1 X 10^8 pfu of the Ad-sig-E7/ecdCD40L Vector on the In Vivo Growth of the E7 Positive TC-1 Cells (Solid Diamonds) and the E7 Negative EL-4 Cell Line (Solid Squares). The size of the subcutaneous tumors was estimated by measuring with calipers in two separate orthogonal directions and then calculating the volume assuming an elliptical shape. Fig. 3D. Use of Tetramers to Measure the Level of E7-Specific CD8+ T Cells in the Spleens

of Ad-sig-E7/ecd/CD40L Vector Immunized C57BL/6 Mice. The spleen cells were harvested 10 days following the completion of two subcutaneous injections 7 days apart of with 1 X 10^8 pfu of the following vectors: Ad-sig-E7/ecdCD40L, Ad-E7/wtCD40L, Ad-wtCD40L, and Ad-sig-ecdCD40L. The T cells were then analyzed for the percentage of $E7_{49-57}$ peptide specific CD8+ T cell lymphocytes by H-$2D^b$ tetramer staining. Fig. 3E. Enzyme-Linked Immuno-Spot (ELISPOT) Assay Show Increase in the Level of Interferon-Alpha (IFN-Alpha) Secreting Cells in the Spleen Cells of Mice Injected Subcutaneously Twice (7 Days Apart) with 1 X10^8 pfu of the Ad-sig-E7/ecdCD40 Vector. Mice were injected two times with the following vectors: Ad-sig-E7/ecdCD40L, Ad-sig-ecdCD40L, Ad-E7/wtCD40L, and Ad-wtCD40L. Splenic T cells taken from the mice one week later were analyzed by ELISPOT assay for the presence of interferon gamma. Fig. 3F. Increase in the Level of E7 Specific CTLs in the Spleens of Ad-sig-E7/ecdCD40L Injected Mice. Mice were injected subcutaneously twice (7 days apart) with 1 X 10^8 pfu of the following vectors: Ad-sig-E7/ecdCD40L, Ad-E7/wtCD40L, Ad-sig-ecdCD40L, Ad-wtCD40L, and control (no vector injection). T cells were harvested from the spleens of the test mice 1 week after the second adenoviral vector injection, and were restimulated in vitro with TC-1. After 7 days, restimulated effector cells (spleen cells exposed to TC-1 cells in vitro) were mixed at varying ratios with TC-1 (E7 positive) and EL-4 (E7 negative) target cells. Then the LDH released from the target cells was then measured. There was no LDH detectable from any of the mixtures of EL-4 and the restimulated effector cells isolated from the vaccinated mice, whereas there were significant levels of LDH released from the TC-1 target cells when they were mixed with

the restimulated effector cells isolated from the mice vaccinated with the Ad-sig-E7/ecdCD40L vector.

Figure 4: The ecdhMUC1 Protein Released from Ad-sig-ecdhMUC1/ecdCD40L Vector Infected Cells Forms Functional Trimers and Activates DCs. Fig. 4A. Induction of Interferon Gamma Secretion from BM-Derived DCs Induced by Exposure to the Ad-sig-ecdhMUC1/ecdCD40L Vector. Supernatant medium collected from DCs derived in vitro from hMUC1.Tg mice following exposure to the Ad-sig-ecdhMUC1/ecdCD40L vector or to the Ad-sig-ecdhMUC1 vector and then analyzed for the levels of interferon gamma. Fig. 4B. Induction of IL-12 Secretion from BM-Derived DCs Induced by Exposure to the Ad-sig-ecdhMUC1/ecdCD40L or to the Ad-sig-ecdhMUC1 Vectors. The same procedure as outlined in Figure 4A was carried out except that the supernatant medium was analyzed for IL-12.Fig. 4C. Non-Denaturing Gel Analysis of Molecular Weights of the ecdhMUC1/ecdCD40L Protein. A construct was created in which a His tag was placed at the carboxyl terminal end of the CD40L and a HSF1 trimeric stabilization domain was added between the ecdhMUC1 and ecdCD40L domains. Following release from vector-infected cells, the protein was purified using a His tag column, concentrated and added to a non-denaturing gel. The protein in the lane labeled "MUC1/CD40L trimer" was added to the non-denaturing gel without treatment. The protein in the lane labeled "MUC1/CD40L monomer" was first treated with the denaturing conditions before loading on the gel. The molecular weight markers are given in the extreme right hand lane.

Figure 5: The Effect of Two Subcutaneous Injections (7 Days Apart) of 1 X 10^8 pfu of the Ad-sig-ecdhMUC1/ecdCD40L Vector on the In Vivo Growth of the hMUC1 Positive LL2/LL1hMUC1 Cancer Cell Line in hMUC1.Tg Mice. Fig. 5A. Two Subcutaneous Injections (7 Days Apart) of 1 X 10^8 pfu of the Ad-sig-ecdhMUC1/ecdCD40L Vector Suppresses the Growth of the Human MUC1 Positive LL2/LL1hMUC1 Cancer Cell Line. The Ad-sig-ecdhMUC1/ecdCD40L vector (solid squares) or the Ad-sig-ecdhMUC-1 vector (solid triangles) was injected twice at 7-day intervals (solid squares) or was not injected with any vector (solid diamonds). One week following the second vector injection, the mice were injected with 5 X 10^5 LL2/LL1hMUC1 cancer cells, which were positive for hMUC1, and the growth of these cells was measured with calipers. Fig. 5B. The Ad-sig-ecdhMUC1/ecdCD40L Induced Suppression is Specific For the hMUC1 Antigen. hMUC1.Tg mice were injected twice subcutaneously (7 Days Apart) with 1 X 10^8 pfu of the Ad-sig-ecdhMUC1/ecdCD40L vector twice at 7-day intervals. One week following the second vector injection, the mice were injected with 5 X 10^5 LL2/LL1hMUC1 cells which are positive for the hMUC1 antigen (solid squares) or the same number of LL2/LL1 cells which are negative for the hMUC1 antigen (solid circles). Fig. 5C. Survival of LL2/LL1hMUC1 Cell Line Injected hMUC1.Tg Mice that were Twice (7 Days Apart) Subcutaneously or Not Vaccinated with 1 X 10^8 pfu of the Ad-sig-ecdhMUC1/ecdCD40L Vector. The mice in Figs. 5A were followed for survival following the injection of the LL2/LL1hMUC1 cells. Continuous bold line: mice injected with the Ad-sig-ecdhMUC1/ecdCD40Lvector; broken bold line: mice not injected with a vector.

Figure 6: Mechanism of the Suppressive Effect of the Ad-sig-ecdhMUC1/ecdCD40L Vector on Induction of the Immune Suppression of the Growth of the LL2/LL1hMUC1 Cells in hMUC1.Tg Mice. Fig. 6A. Subcutaneous Injection of the ecdhMUC1/ecdCD40L Protein Does Not Induce Suppression of the Growth of hMUC1 Positive Cells Which is Equivalent to That Seen with Two Subcutaneous Injections of 1 X 10^8 pfu of the Ad-sig-ecdhMUC1/ecdCD40L Vector. 500,000 LL2/LL1hMUC1 cells were injected subcutaneously into the hMUC1.Tg mice. Then, two days following the injection of the tumor cells, the ecdhMUC1/ecdCD40L protein was injected subcutaneously into hMUC1.Tg mice. Solid Diamonds: No protein injection. Solid Squares: Ad-sig-ecdhMUC1/ecdCD40L vector. Open Circles: two injections of the ecdhMUC1/ecdCD40L protein; Solid Squares with the X: One injection of the ecdhMUC1/ecdCD40L protein. Fig. 6B. CD4 Depleted T Cells from hMUC1 Transgenic Mice Following Two Subcutaneous Injections of 1 X 10^8 pfu of the Ad-sig-ecdhMUC1/ecdCD40L Vector Secrete Increased Levels of Interferon Gamma. CD8+ T cells were isolated from hMUC1.Tg mice which had been vaccinated two times with the Ad-sig-ecdhMUC1/ecdCD40L vector or with the Ad-sig-ecdhMUC-1 vector, or which had been unvaccinated (labeled "no vector"). 7 days after the vaccination, the CD8 cells harvested from the spleens of the test animals and then were incubated for 24 hours. The supernatant medium was analyzed for the levels of interferon-gamma. Fig. 6C. Cytotoxicity of CTLs From hMUC1.Tg Transgenic Mice Following Two Subcutaneous Injections (7 Days Apart) of 1 X 10^8 pfu of the Ad-sig-ecdhMUC1/ecdCD40L Vector Against LL2/LL1-MUC1 hMUC1 Positive Cancer Cells or Against LL2/LL1 Cancer Cells which are Negative for the hMUC1 Antigen. CD8+ T cell lymphocytes were

isolated from the spleens of hMUC1.Tg mice one week following vaccination with the Ad-sig-ecdhMUC1/ecdCD40L vector. The cells were restimulated in vitro with the LL2/LL1hMUC1 cell line for 5 days (solid diamonds) or the LL2/LL1 cell line (solid squares). CD8+ T cell lymphocytes were also isolated from the spleens of hMUC1.Tg mice one week following vaccination with the Ad-sig-ecdhMUC1 vector which was then stimulated in vitro with the LL2/LL1hMUC1 cell line (solid triangles). Then, the CD8 cells from the vaccinated animals were mixed with either the LL2/LL1hMUC1 cell line (solid diamonds) or the hMUC1 negative LL2/LL1 parental cell line (solid squares) in different effector/target ratios (20/1, 10/1 and 5/1). The LDH released from each of these cell mixtures (ordinate) was then measured. Fig. 6D. Phosphorylation of the ERK1/ERK2 Proliferation Pathway in CD8 T Cells from hMUC1 Transgenic Mice Following Stimulation with Bone Marrow Derived DCs Infected with the Ad-sig-ecdhMUC1/ecdCD40L Vector. CD8 T cells were isolated by CD4 depletion from the spleen cells of hMUC1.Tg mice one week following the completion of two subcutaneous injections (one week apart) with the Ad-sig-ecdhMUC1/ecdCD40L vector (Panel A) or from mice which were not vaccinated (Panel B). DCs, which had been infected with the Ad-sig-ecdhMUC1/ecdCD40L vector, were then mixed in a 1/1 ratio with the restimulated CD8+ T cells. Proteins were isolated from these mixtures 0, 5, 15, and 45 minutes later and separated on a SDSPAGE, transferred to a Western filter by blotting, and then analyzed for phosphorylation of the P44 and P42 MAK proteins using the New England BioLabs kit for phosphorylated proteins. The blot for the vaccinated mice is shown in Panel A and the blot for the unvaccinated mice is shown in Panel B.